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14. ABSTRACT

This project was part of an Amyotrophic Lateral Sclerosis Research Program - Therapeutic Development Award with a 3-year period of performance. An Extension With-Out Funds (EWOF) was granted in Year 4 for the continuation of data analysis. The project intended to refine critical details required for successful cell transplantation.
Aim 1 (Optimal Surgical Technique) provided critical data on tolerance and toxicity of cell dosing and numbers of permissible spinal cord injections.
Aim 2 (Graft Rejection) is still being analyzed and will generate data on optimization of immunosuppression following spinal cord stem cell transplantation.

15. SUBJECT TERMS
ALS, spinal cord, stem cell, swine, therapy, transplantation.

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INTRODUCTION:

This project was part of an Amyotrophic Lateral Sclerosis Research Program - Therapeutic Development Award with a 3-year period of performance. An Extension With-Out Funds (EWOF) was granted in Year 4 for the continuation of data analysis. Due to the volume of generated material and the complexity of methodologies applied, data analysis is still ongoing and final conclusions pertaining to Aim 2 have been delayed.

KEYWORDS: ALS, cell therapy, escalation, graft, number, pigs, safety, spinal cord, swine, tolerance, transplantation, volume.

OVERALL PROJECT SUMMARY:

The present application intended to refine critical details required for successful cell transplantation.
Aim 1 (Optimal Surgical Technique) provided critical data on tolerance and toxicity of cell dosing and numbers of permissible spinal cord injections.
Aim 2 (Graft Rejection) is still being analyzed and will generate data on optimization of immunosuppression following spinal cord stem cell transplantation.

KEY RESEARCH ACCOMPLISHMENTS:

In **Year 1** (Sep 30, 2011 – Sep 29, 2012) we completed all surgical procedures pertaining to Aim 1 (Optimal Surgical Technique - Number-Escalation, Volume-Escalation, and Comparison of Cannulas) and generated a video-protocol manuscript describing our surgical technique (*Appendix 1*).
At that point, based on our experience injecting the human spinal cord, we concluded that it was unnecessary to pursue Aims 1d (Uni *vs*. Bilateral Injections) and 1e (Staggered *vs*. Symmetric Injections).
A total of 45 animals were used.

Aim	Brief Explanation	Number Pigs	Number Injections	Volume Injections	Cell type	Immunosuppression	Survival
1a	Number-escalation	7	20	10	Human NPCs	Tacrolimus (0.025 mg/kg, BID, IV)	21d
		5	30				
		5	40				
1b	Volume-escalation	7	20	10	Human NPCs	Tacrolimus (0.025 mg/kg, BID, IV)	21d
		5		25			
		5		50			
		2		75			
1c	Hand-held Cannula	3	20	10	Human NPCs	Tacrolimus (0.025 mg/kg, BID, IV)	21d
		3		25			
		3		50			

In **Year 2** (Sep 30, 2012 – Sep 29, 2013) we generated the initial histological data pertaining to Aim 1. We also completed all surgical procedures pertaining to Aims 2a and 2c (Graft Rejection – Allografts *vs*. Xenografts, Mono *vs*. Triple Immunosuppression) using human and pig NPCs provided by the Svendsen's lab*. Because generation of porcine iPS-derived NPCs was not possible, we had to drop Aim 2b (Graft Rejection – Autografts).
A total of 30 animals were used.

Aim	Brief Explanation	Number Pigs	Number Injections	Cell type	Immunosuppression	Survival
2a	Allograft vs. Xenograft	5	5	Pig NPCs	None	21d
		5			Tacrolimus (0.025 mg/kg, BID, IV)	
		5		Human NPCs	None	
		5			Tacrolimus (0.025 mg/kg, BID, IV)	
2c	Mono vs. Triple Immunotherapy	5	5	Pig NPCs	Basiliximab (two 10mg IV doses on post-op day 0 and day 4) + MMF (10 mg/kg, BID, IV) + Tacrolimus (0.025mg/kg, BID, IV)	21d

** Human and Pig NPCs from Svendsen Lab: Following transplantation, human cells can easily be identified with the use of human specific antibodies. However, as allogeneic grafts, the transplanted pNPCs can only be identified following transplantation by means of an exogenous marker. In this instance, they selected to label proliferating cells in vitro via pulsing with 5-bromo-2'-deoxyuridine (BrdU). Although toxic at high concentrations (Caldwell et al., 2005) they have found that pulsing cells with BrdU at a concentration of 0.2µM or lower is non-toxic and a reliable labeling strategy for allogeneic transplant or assessment of cell proliferation (Suzuki et al., 2006; Ebert et al., 2010). For this study, as the pNPCs are slow growing cells, they found that they obtained the best results by pulsing for 3 days with 0.05µM BrdU. This paradigm resulted in the labeling of 79% and up to 45% of human and pig NPCs respectively. Using these parameters they provided 9 vials of BrdU pulsed hNPCs (passage 29) and 13 vials pNPCs (passage 12) to our group for transplantation in Aims 2a and 2c. Other attempts (2 attempts) at thawing and expanding pNPCs to provide a greater number of cells for the Boulis group have not been successful. They were unsure of the factors causing the rapid senescence of the pNPCs. However, low expansion rates and viability of cells post-thaw have resulted in these cells not being suitable for transplantation.*

In **Year 3** (Sep 30, 2013 – Sep 29, 2014) we completed histological and stereological analyses pertaining to Aim 1 and began the preparation of 2 manuscripts reporting our findings. We also began the analysis of peripheral blood for the presence of graft-specific host antibodies (animals from Aim 2a). Moreover, we initiated surgical procedures pertaining to Aims 2d and 2e. A total of 20 surgical procedures were performed.

In Feb 2014, we received approval for the termination of subcontract, as the former subcontract (Svendsen laboratory at Cedar Sinai) could not fulfill their original plan of delivering pig iPS-derived NPCs and fetal *cortical*-derived pig NPCs or to conduct part of the tissue analysis, as originally proposed. Preliminary data analysis of tissue from Aims 2a-c has revealed sub-optimal engraftment / survival of

BrdU-labeled pNPCs in contrast to hNPCs (**Figure 1**). As an alternative, we established collaboration / agreement with Neuralstem, Inc. to utilize their GFP-expressing fetal *spinal cord*-derived pig NPCs to complete Aims 2d and 2e (**Figure 2**).

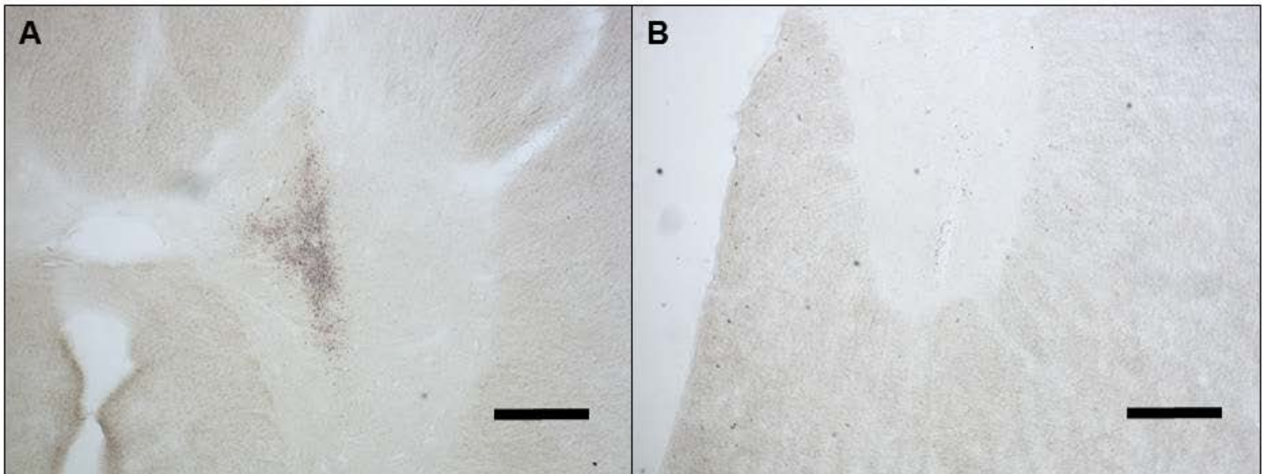

Figure 1. Characterization of the immune response to transplantation in the spinal cord using Xenograft vs. Allograft models. A. Successful identification of transplanted human neural progenitor cells using a human nuclear antigen (HuNu) antibody, **B.** Unsuccessful identification of BrdU-labeled porcine neural progenitor cells using a specific antibody for BrdU. Scale bars = 500 microns.

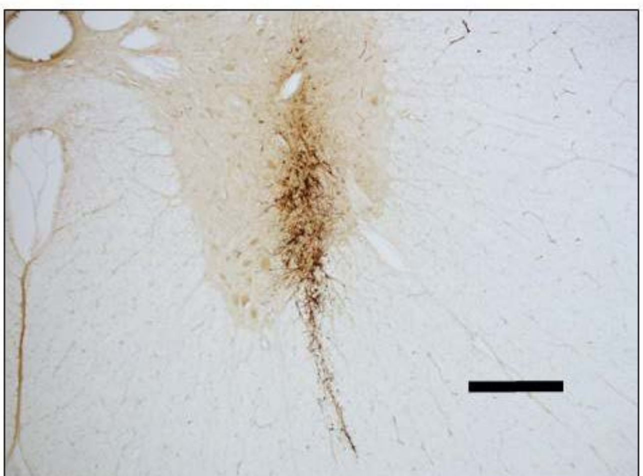

Figure 2. Sensitization of the immune system to transplantation in the spinal cord using an allograft model. Successful identification of transplanted swine neural progenitor cells labeled with GFP using a GFP-specific antibody. Scale bars = 500 microns.

Originally, in Aims 2d and 2e animals would receive immunosuppressant treatment with the best regimen defined in <u>Aim 2c</u>. However, due to the change in source of cells for Aims 2a-c and Aims 2d-e, we were not able to establish a <u>relevant</u> correlation between data from these sub-Aims and chose to proceed with monotherapy (Tacrolimus) in Aims 2d-e.

Aim	Brief Explanation	Number Pigs	Number Injections	Cell Type	Immunosuppression	Survival
2d	Repeated Injections (Sensitization)	5	10L + 10C	Pig NPCs + Pig NPCs	Tacrolimus (0.025 mg/kg, BID, IV)	21d + 21d
		5	10L + 10C	Vehicle + Pig NPCs		
2e	Immunosuppression Withdrawal	5	10L	Pig NPCs	None	42d
		5			Tacrolimus for 14 days	
		5			Tacrolimus for 42 days	

C = cervical - L = lumbar

In **Year 4** (*EWOF* Sep 30, 2014 – Sep 29, 2015 and present) we generated 2 manuscripts (1 published and 1 under 2nd review) with data from Aim 1. We also concluded surgical procedures and initiated histological analyses pertaining to Aim 2. A total of 15 surgical procedures were performed. A total of 25 animals were used.

Due to the volume of generated material and the complexity of methodologies applied (for instance, certain antibodies with specificity for pig tissue had to have their protocols optimized), data analysis is still ongoing and final conclusions pertaining to Aim 2 have been delayed. In addition to GFP for graft identification, immunohistochemistry for the following markers (Iba-1, cd4, and cd8) has been recently performed. We expect to conclude analyses and preparation of manuscripts in the next 6 months.

The following preliminary data pertaining to Aim 2 has been generated so far (**Figures 3 and 4**):

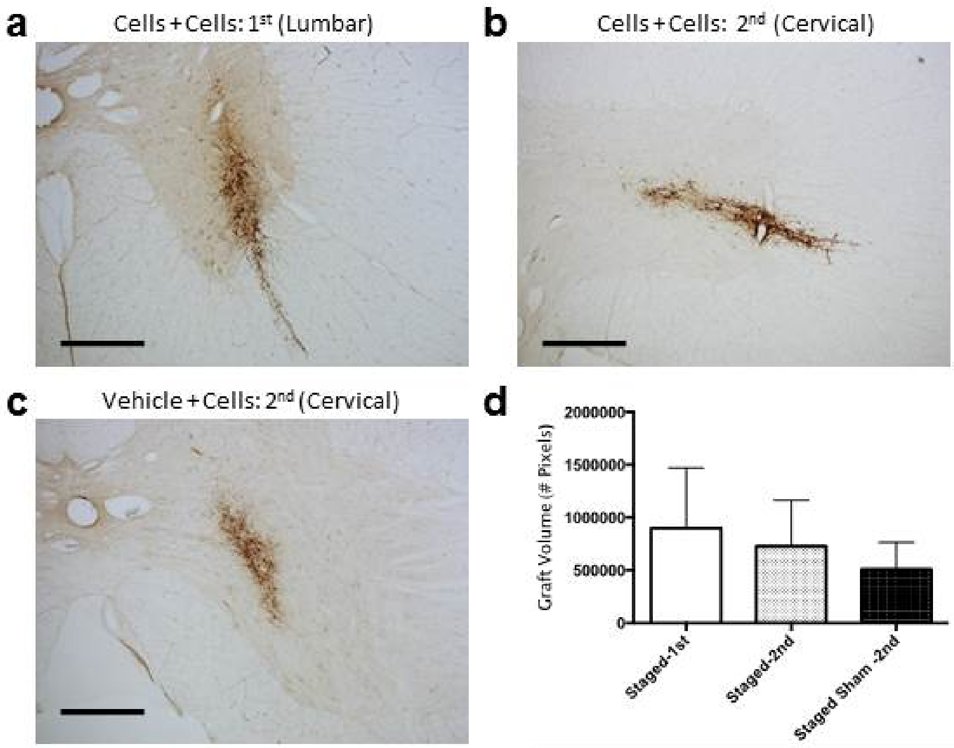

Figure 3. Quantification of Transplanted Cell Graft Survival – Aim 2d. Representative micrographs of immunohistochemical staining with a mouse monoclonal anti-Green Fluorescent Protein (GFP) in pig Neural Progenitor Cell (pNPC) grafts are shown. The first cohort of animals received pNPC grafts in the lumbar spinal cord (**a**) followed by additional pNPC grafts in the cervical spinal cord (**b**) three weeks after the original lumbar transplantation (cells + cells cohort). The second cohort of animals received vehicle injection in the lumbar spine (not shown) followed by pNPC grafts in the cervical spinal cord (**c**) three weeks after lumbar injection (vehicle + cells cohort). The pigs received Tacrolimus immunosuppression (Prograf, 0.025 mg/kg, BID, IV) for the six week duration of the study. GFP+ signal was not observed on the contralateral side, in control tissue, or in vehicle injection sites. GFP+ graft volume was calculated for all groups (**d**). Graft volume was calculated using a color threshold method with ImageJ. Scale bars: 1 mm. Statistical analyses were performed using a standard one-way ANOVAs with Tukey's post-hoc multiple comparisons. *Significant, $P < 0.05$; **Significant, $P < 0.005$; ***Significant, $P < 0.0005$. GraphPad prism software was used to determine significance and generate graphs. Graphs displayed as mean ± SD.

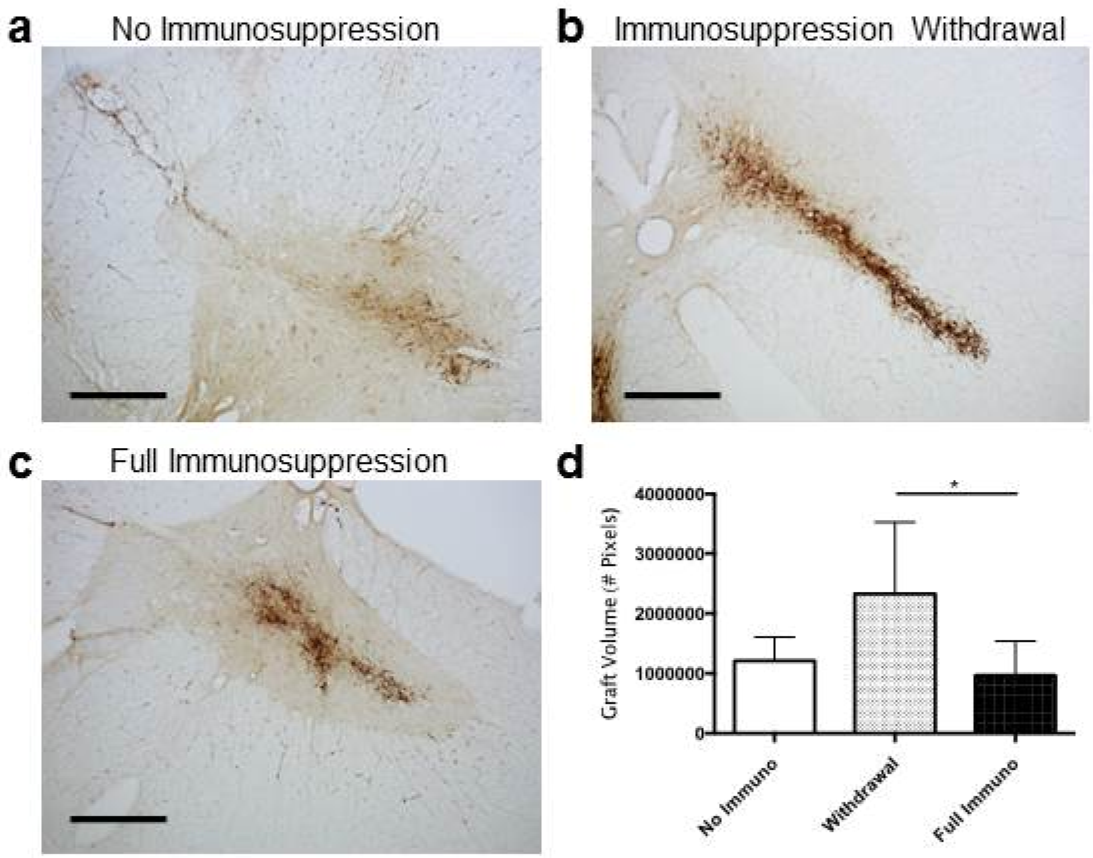

Figure 4. Quantification of Transplanted Cell Graft Survival – Aim 2e. Representative micrographs of immunohistochemical staining with a mouse monoclonal anti-Green Fluorescent Protein (GFP) in pig Neural Progenitor Cell (pNPC) grafts from animals that received no immunosuppression (**a**), immunosuppression for the first two weeks after transplantation (**b**), and immunosuppression for the six-week duration of the study (**c**). The immunosuppression agent used was Tacrolimus (Prograf, 0.025 mg/kg, BID, IV). GFP+ signal was not observed on the contralateral side or in control tissue. GFP+ graft volume was calculated for all groups (**d**). Graft volume was calculated using a color threshold method with ImageJ. Scale bars: 1 mm. Statistical analyses were performed using a standard one-way ANOVAs with Tukey's post-hoc multiple comparisons. *Significant, $P < 0.05$; **Significant, $P < 0.005$; ***Significant, $P < 0.0005$. GraphPad prism software was used to determine significance and generate graphs. Graphs displayed as mean ± SD.

CONCLUSIONS:

Aim 1:

- In Aim 1, **all animals recovered to baseline behavior** by post-operative day 14 despite the increasing number of injections and volume.
- The severity of **acute transient morbidity should not be neglected**, as well as the observation that the cord acutely swells with volume escalation making dural closure virtually impossible.
- The higher the number of injections the more likely is that the vasculature will interfere with a symmetric / linear pattern of injections. Staggered injections and reduced interval spacement of injections become a necessity.
- Hand-held injections can be performed in an incredibly quicker manner in comparison with injections using the platform. Nonetheless, accuracy and reflux are the main disadvantages of such technique.
- Our statistical analyses of volume escalation indicate that **25 microliters is likely the optimal volume to inject** with the cell concentration used in these experiments.
- The enormous variability in-between and with-in groups can explain high variance in the data.
- The engraftment percentage achieved in the completed animals is slightly higher compared to what has been reported in the literature.

In summary, the optimal injection paradigm should use an injection volume between 10 and 25 uL at a fixed concentration of 10,000 cells/uL. Furthermore, the number of injections can be safely increased to a total of 40 when using a volume of 10 uL (**Figure 5**). These safety thresholds should be interpreted with caution when choosing an injection strategy for other applications or diseases. It is also important to account for other factors such as anatomic location, underlying disease, patient characteristics, cell type and concentration, delivery vehicle, and infusion rate.

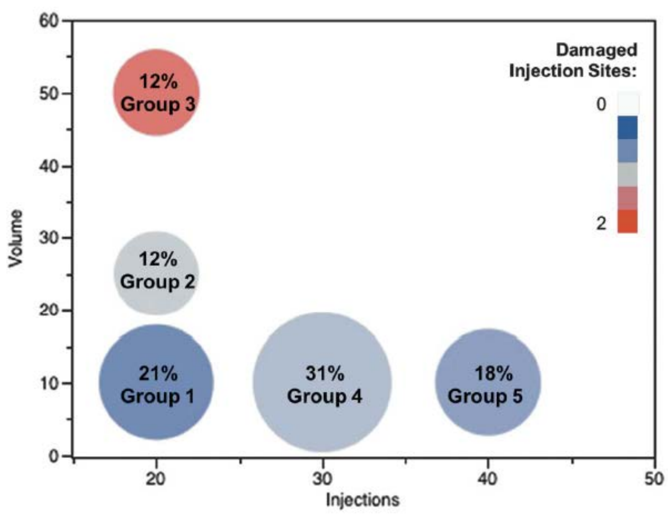

Figure 5. *This graph shows that as injection volume increases, the number of damaged injection sites increases and the engraftment percentage remains similar among groups (12%-21%). Additionally, it shows that as the number of injections increases, tissue damage stays relatively the same, but at 30 injections, the best engraftment percentage is achieved (31%). It is important to note that increased trauma results in increased local inflammatory and immune response; thus, this might be a potential explanation for why a greater engraftment percentage was observed with 30 injections compared with 40 injections.*

Aim 2:

Flow Characterization:

- **Both human and pig NPCs express MHC Type 1 and 2 antigens**. This allows them to directly present self and non-self antigens to the host immune system.
- **A transient increase in graft-specific antibodies was detected 7 and 14 days post-operatively in pigs that did not receive immunosuppression.** No increase was observed in the tacrolimus group. Different patterns of antibody response were observed in pigs in the no immunosuppression cohort, with both "responders" and "non-responders".
- **A trend showing a slight decrease in graft-specific antibodies was observed in the clinical trial patients**.

"PUBLICATIONS, ABSTRACTS AND PRESENTATIONS":

Manuscripts

1. Federici T, Hurtig CV, Burks KL, Riley JP, Krishna V, Miller BA, Sribnick EA, Miller JH, Grin N, Lamanna JJ, Boulis NM. (2012) Surgical Technique for Spinal Cord Delivery of Therapies. Demonstration of Procedure in Gottingen Minipigs. JoVE. e4371.
   Appendix 1

2. Gutierrez J, Lamanna JJ, Grin N, Hurtig CV, Miller JH, Riley J, Urquia L, Avalos P, Svendsen CN, Federici T, Boulis NM. (2015) Preclinical Validation of Multilevel Intraparenchymal Stem Cell Therapy in the Porcine Spinal Cord. Neurosurgery. 2015 Oct; 77(4):604-12.
   Appendix 2

3. Gutierrez J, Moreton CL, Lamanna JJ, Schapiro R, Grin N, Hurtig CV, Miller JH, Riley J, Urquia L, Federici T, Boulis N. Understanding Cell Distribution after Direct Transplantation into the Spinal Cord: A Tool to Determine the Optimal Transplantation Volume. *Under Review*.
   Appendix 3

4. Lamanna JJ, Gutierrez J, Espinosa JR, Urquia L, Grin J, Hurtig CV, Riley J, Bordeau J, Polak M, Brannon P, Glass J, Federici T, Kirk AD, Boulis NM. FK-506 immunosuppression inhibits production of systemic graft-specific antibodies in a large animal xenograft model of intraspinal stem cell transplantation. *In Preparation*.

Abstracts

1. Quach DM, Boulis NM, Federici T, Hefferan MP, Wu S, Lee D, Lam T, Hayama KL, Hazel T, Marsala M, Wu MC, Johe K. Immune Tolerance after Cervical Transplantation of a Human Neural Stem Cell Line (NSI-566RSC) in Naïve Minipigs. Society for Neuroscience Abstract 555.27, 2012.

2. Miller JH, Hurtig CV, Grin N, Lamanna JJ, Federici T, Boulis NM. Porcine behavior and functional status following large volume cervical spinal cord injections. Abstract for AANS/CNS Spine Section meeting: Phoenix, AZ March 6-9, 2013.

3. Lamanna JJ and Espinosa J, Gutierrez J, Urquia L, Grin N, Federici T, Kirk AD, Boulis NM. Peripheral blood detection of a graft-specific immune response to intraspinal stem cell therapy in Gottingen minipigs. Abstract for American Association of Neurological Surgeons Annual Meeting: San Francisco, CA April 5-9, 2014.

4. Gutierrez J, Lamanna JJ, Grin N, Hurtig CV, Miller JH, Riley J, Urquia L, Federici T, Boulis NM. Preclinical Validation of Multilevel Intraspinal Stem Cell Therapy for Amyotrophic Lateral Sclerosis (ALS). Congress of Neurological Surgeons Annual Meeting (Boston, October 2014).

5. Lamanna JJ, Gutierrez J, Espinosa JR, Urquia L, Grin J, Hurtig CV, Riley J, Bordeau J, Polak M, Brannon P, Glass J, Federici T, Kirk AD, Boulis NM. Peripheral Monitoring of Immune Response to Intraspinal Stem Cell Therapy. Congress of Neurological Surgeons Annual Meeting (Boston, October 2014).
   Appendix 4

6. Gutierrez J, Moreton CL, Lamanna JJ, Schapiro R, Grin N, Hurtig CV, Miller JH, Riley J, Urquia L, Federici T, Boulis N. Understanding Cell Migration after Direct Transplantation into the Spinal Cord: A Tool to Determine the Optimal Transplantation Volume. Congress of Neurological Surgeons Annual Meeting (New Orleans, September 2015). *Neurosurgery* 62 (2015): 234-234.

"INVENTIONS, PATENTS AND LICENSES":

N/A.

REPORTABLE OUTCOMES:

Data generated from Aim 1 demonstrates that although increasing number of injections up to 40 in the porcine spinal cord did not result in neurological morbidity or tissue damage, greater number of injections at a constant volume of 10ul would require considerably longer surgical procedures (*Appendix 2*).

Data generated from Aim 1 also demonstrates that although the total volume of grey matter occupied by higher volumes of stem cell grafts was significantly larger when compared to 10ul grafts (*Appendix 3*), tissue damage was identified when injecting larger volumes, such as 50ul (*Appendix 2*).

Data generated from Aim 2a (monotherapy vs. no immunosuppression following hNPCs transplantation) provides evidence for a decreased immune response to transplanted intraspinal stem cell grafts with immunosuppression (*Appendix 4*).

OTHER ACHIEVEMENTS:

Data generated in Aim 1 has been **“**disseminated to communities of interest” and consequently assisted other research groups (Svendsen group, Q Therapeutics) in their planning for upcoming trials and IND submissions to the FDA for ALS indication.

Q Therapeutics has now received FDA clearance and is transitioning to clinical development, with a first-in-human Phase 1/2a clinical trial planned in patients with amyotrophic lateral sclerosis (ALS, or Lou Gehrig’s disease).

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LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT:

Name:	Nicholas Boulis, MD
Project Role:	Principal Investigator
Contribution to Project:	Principal Investigator
Effort:	3%

Name:	Thais Buchman, PhD
Project Role:	Key Personnel – Project Manager
Contribution to Project:	Project Manager / Histology / Data Analyses
Effort:	25%

Name:	Natalia Grin (former employee)
Project Role:	Veterinary Technician
Contribution to Project:	Surgical Assistant / Animal Treatment and Care / Histology
Effort:	50%

Name:	Juanmarco Gutierrez, MD
Project Role:	MS Student / Lab Associate
Contribution to Project:	Surgeon / Stereology / Data Analysis / Manuscript Author
Effort:	100%

Name:	Lindsey Urquia
Project Role:	Laboratory Assistant, Senior
Contribution to Project:	Surgical Assistant / Animal Treatment and Care / Histology
Effort:	100%

Name:	Jason Lamanna
Project Role:	MD/PhD Student / Lab Associate
Contribution to Project:	Cell preparation / Surgeon / Immunology / Data Analysis / Author
Effort:	35%

Name:	Cheryl Moreton
Project Role:	Volunteer
Contribution to Project:	Histology / Stereology
Effort:	25%

APPENDICES:

Appendix 1

Appendix 2

Appendix 3

Appendix 4

Video Article

Surgical Technique for Spinal Cord Delivery of Therapies: Demonstration of Procedure in Gottingen Minipigs

Thais Federici[1], Carl V. Hurtig[1], Kentrell L. Burks[1], Jonathan P. Riley[1], Vibhor Krishna[2], Brandon A. Miller[1], Eric A. Sribnick[1], Joseph H. Miller[3], Natalia Grin[1], Jason J. Lamanna[1,4,5], Nicholas M. Boulis[1]

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URL: http://www.jove.com/video/4371/
DOI: 10.3791/4371

Keywords: accuracy, delivery, safety, spinal cord, target, therapy, transplantation

Date Published: 9/14/2012

Citation: Federici, T., Hurtig, C.V., Burks, K.L., Riley, J.P., Krishna, V., Miller, B.A., Sribnick, E.A., Miller, J.H., Grin, N., Lamanna, J.J., Boulis, N.M. Surgical Technique for Spinal Cord Delivery of Therapies: Demonstration of Procedure in Gottingen Minipigs. *J. Vis. Exp.* (), e4371 10.3791/4371, DOI : 10.3791/4371 (2012).

Abstract

This is a compact visual description of a combination of surgical technique and device for the delivery of (gene and cell) therapies into the spinal cord. While the technique is demonstrated in the animal, the procedure is FDA-approved and currently being used for stem cell transplantation into the spinal cords of patients with ALS. While the FDA has recognized proof-of-principle data on therapeutic efficacy in highly characterized rodent models, the use of large animals is considered critical for validating the combination of a surgical procedure, a device, and the safety of a final therapy for human use. The size, anatomy, and general vulnerability of the spine and spinal cord of the swine are recognized to better model the human. Moreover, the surgical process of exposing and manipulating the spinal cord as well as closing the wound in the pig is virtually indistinguishable from the human. We believe that the healthy pig model represents a critical first step in the study of procedural safety.

Video Link

The video component of this article can be found at http://www.jove.com/video/4371/

Protocol

1. Animal Use

Procedures demonstrated herein have been approved by the Emory University Institutional Animal Care and Use Committee (IACUC). Female Gottingen minipigs weighing approximately 15-20 kg are used.

2. Anesthesia

Animals are fasted approximately 12 hours prior to surgery. Animal sedation and anesthesia induction consist of a cocktail of intramuscular Ketamine (35 mg/kg), Acepromazine (1.1 mg/kg), and Atropine (0.04 mg/kg). Animals are then intubated and maintained on oxygen and 1-3% isoflurane general anesthesia. At this point, the back and head of each animal is shaved. Depth of anesthesia is monitored by the veterinary staff. Absence of interdigital, corneal and palpebral reflexes, as well as heart and respiratory rate, pulse oximetry, direct/indirect blood pressure, end-tidal carbon-dioxide measurements, and muscle tone/response to noxious stimuli are used to monitor depth of anesthesia.

3. Positioning

Animals are taken to the operating room and placed into a prone position on a frame custom designed to mimic patients positioning on a Jackson spinal surgical table. The frame utilizes adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free and, therefore, minimizing pressure on the abdomen and chest and consequent epidural venous bleeding (**Figure 1**). The frame also provides external immobilization of the spine for the procedure [1].

Additionally, animals are placed on a heated re-circulating pad to maintain body temperature and a marginal ear vein catheterized for fluid administration and any necessary drug delivery during surgery. Finally, the surgical field is prepped with alcohol and Chlorhexadine or Betadine solution and surgical drapes are placed on the surgical field.

4. Laminectomy

An approximately 10-15 cm skin incision is performed and the paraspinal musculature is dissected off the spine bilaterally. Next, a dorsal multi-level laminectomy is performed. The lamina and spinous processes of three vertebrae overlying C3-C5 or L2-L4 segments are removed using rongeurs and a surgical drill.

5. Placement of the Spinal Derrick

We call Spinal Derrick the device designed for the delivery of (gene and cell) therapies into the spinal cord [2-5]. Detailed discussion on the design and evolution of this device can be found on Riley *et al*., 2011. [4]

To secure the device to the patient, percutaneous posts are placed through 1cm skin incisions above and below the primary incision and mounted to the lamina above and below the primary incision.

Next, two integrated retractors are attached to the four percutaneous posts above and below the incision site to expose the area of the spine that has undergone laminectomy.

6. Dural Opening

With the aid of a Woodson dental tool and an 11 blade, a 2.5 cm incision is made through the dura, exposing the spinal cord. The dura is reflected away from the pial layer using 4-0 Nurolon suture and secured to the deep paraspinal musculature.

Surgical patties are placed in the rostral and caudal extremes of the opening. These provide a partial barrier to cerebrospinal fluid flow and also provide a safe target for the surgeons to place suckers without damaging the cord. In humans, under surgical microscope magnification, the pial surface is dissected at this point. Due to technical limitations, this procedure is not required or feasible in animals.

7. Spinal Cord Injections and Lateral Displacement of the Spinal Derrick for Additional Injections

Immediately prior to injections, a bolus of Methylprednisolone (125 mg, IV) is given to prevent spinal cord swelling.

At this point, the platform rail system is attached and the side rails are adjusted to fit the appropriate length. The gondola is top-loaded onto the 2 bars and the Z drive is mounted on the universal joint. Next, the loaded cannula is placed onto the microdrive. Using the universal joint on the microinjection platform, the coronal and sagittal angles are adjusted to ensure a trajectory orthogonal to the surface of the spinal Injections follow the placement of the cannula. The needle is positioned accordingly medial to the dorsal root entry zone (DREZ). The DREZ is identified under 3.5X surgical loupe magnification and penetrated on an orthogonal trajectory to the cord surface at a point <1 mm medially.

In humans, a pre-operative MRI provides a baseline assessment of spinal cord dimensions for operative planning. Moreover, the thickness of the spinal cord is measured to determine the target depth of the ventral horn.

The suspension is infused at a depth of 4 mm from pial contact. A flange made of ultem plastic serves as a stopper on the pia surface to prevent the needle from advancing deeper than desired. Once the needle tip is positioned at the target, the rigid metal outer sleeve is pulled up, leaving the flexible tubing exposed. Once the injection is completed, the needle is left in place for an additional 1 min to prevent cell reflux up the cannula injection tract.

Care is taken to avoid surface vasculature by slightly adjusting the microdrive either laterality or rostro-caudally. Some bleeding from penetration sites may occur. When such bleeding is encountered, micro patties are placed over the bleeding puncture site and suction is applied to them to wick blood out of the cannula penetration site and prevent buildup in the cord. This reliably allows for the blood to coagulate. Cautery is avoided as is direct pressure.

Following needle removal, the stereotaxic apparatus is relocated to the next target site along the rostro-caudal axis, separated by 2 or 4 mm or as necessary to avoid visible blood vessels on the dorsal surface of spinal cord. This process is repeated as many times as proposed in a given study.

8. Floating Cannula

A custom infusion cannula of narrow diameter is used for the injections. The cannula consists of a 30-gauge beveled needle of fixed length connected to a 30-gauge flexible silastic tubing of variable length. The distal end is fitted with a Hamilton luer lock that is attached to a microinjector pump. The proximal silastic tubing is ensheathed within a 24-gauge rigid outer cannula that seats on the proximal end of the injection needle flange. This flange both seats the outer cannula and serves as a depth stop for the injection needle. For each injection, the appropriate volume of a therapeutic suspension is infused by a using a pre-calibrated MINJ-PD microINJECTOR pump (Tritech Research, Inc., Los Angeles, CA) at a rate of 5 µl per minute.

9. Closure

Once all injections have been made, the spinal derrick is gently removed and the incisions are closed in four layers. The dura is closed using a 4.0 Nurolon stitch, in a watertight fashion. 0 Vicryl suture is used for the deep muscular layer. Fascia is then closed with 0 Vicryl suture also in a watertight fashion. The dermal layer is finally closed with 2.0 Vicryl, with a running stitch. Skin closure is completed using a 3-0 Nylon suture.

10. Recovery and Pain Management

Animals are extubated and monitored for two hours following anesthesia recovery. Next, animals are transferred to individual cages and monitored at least once daily for food consumption, defecation, and micturition.

For pain management, a transdermal Fentanyl patch (75 mcg) is stapled on the back of the animals for three days of post-operative analgesia. Additionally, Buprenorphine (0.05 mg/kg, BID, IM) can also be given for up to three days post-operatively.

11. Results and Representative Outcomes

Clinical and behavior observations are performed before surgery and then recorded on Days 1 through 7 and weekly until endpoint according to the study design. Behavioral data is collected to assess neurological morbidity as previously described [6]. Sensory function is assessed by presence or absence of a withdrawal response to mechanical stimulus to the toes of front and hind limbs. Motor function follows the Tarlov score (**Table 1**): 0 - Paralysis, no movement; 1- Perceptible tonus in the hind limbs, slight movement; 2 - Movement in the hind limbs, but unable to sit or stand; 3 - Ability to stand and walk but ataxic and for short periods; 4 - Complete recovery, normal motor function.

Safety of the procedure is determined by the ability of an animal to return to pre-operative baseline. Transient neurological deficits should mostly resolve between post-operative days 1 and 7, with some variations depending on animals' breeds and procedure (number of injections, among other parameters). Permanent morbidity is defined by lasting neurological deficits which do not resolve by the time animals reach IACUC default endpoint (**Figure 2**).

Representative Results

Tarlov Score	
0	Paralysis, no movement
1	Perceptible tonus in the hind limbs, slight movement
2	Movement in the hind limbs, but unable to sit or stand
3	Ability to stand and walk but ataxic and for short periods
4	Complete recovery, normal motor function

Table 1. ***Tarlov Score.*** Neurological morbidity and recovery is assessed by scoring the animal's motor function.

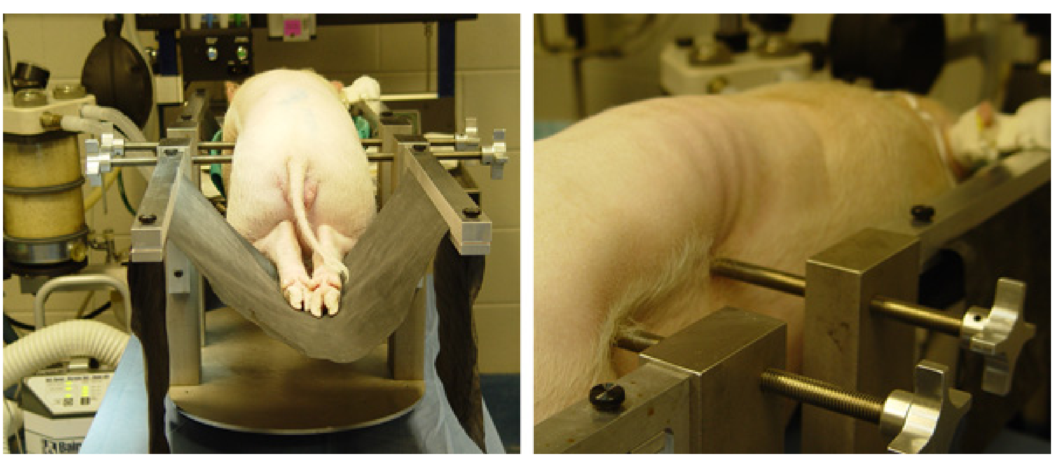

Figure 1. ***Table Positioning for Procedure.*** Animals are placed into a prone position on a frame custom designed to mimic patients positioning on a Jackson spinal surgical table. The frame utilizes adjustable slings that are placed under the chest and pelvis of the animal, allowing the abdomen to hang free and, therefore, minimizing pressure on the abdomen and chest and consequent epidural venous bleeding. The frame also provides external immobilization of the spine for the procedure.

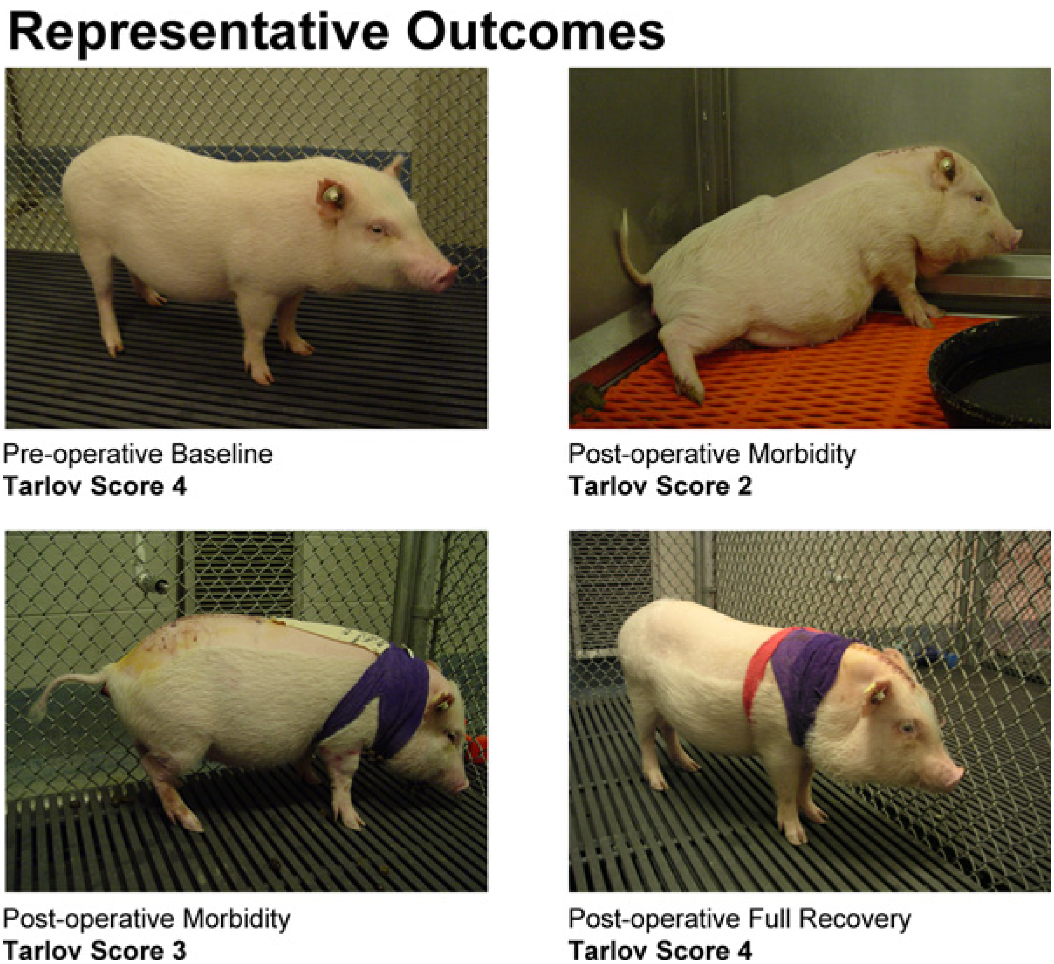

Figure 2. *Motor Function Assessment and Representative Outcomes.* Animals undergo a general neurological examination before surgery and on a regular basis following complete recovery from the procedure. Gait and motor function are assessed according to the Tarlov score. This scale provides objective criteria evaluating the animals' ability to ambulate as a surrogate measure of motor function. Safety of the procedure is determined by the ability of an animal to return to pre-operative baseline. Transient neurological deficits should mostly resolve between post-operative days 1 and 7, with some variations depending on animals' breeds and procedure (number of injections, among other parameters). Permanent morbidity is defined by lasting neurological deficits which do not resolve by the time animals reach IACUC default endpoint.

Supplemental

In case of cell therapies, prior to laminectomy and under anesthesia, a jugular vein 10F chronic catheter (Access Technologies, CCPS072106A) is placed for intravenous administration of immunosuppressants for the duration of the study. The neck of the animal is prepped and draped. The internal jugular vein is exposed surgically and cannulated with the catheter, which is secured with a 3-0 silk tie. The proximal end of the internal jugular is then ligated with a 3-0 silk tie. Next, the catheter is tunneled out of the neck skin dorsally and secured with 3-0 nylon stitches. Finally, the wound is irrigated and closed with a running 3-0 nylon stitch. Such procedure is not required in humans.

Discussion

Despite approval to proceed with the described technique in humans [7-9], critical questions remain to be answered in order for spinal cord therapies to succeed. A rigorous understanding of *spinal cord tolerance* to intraparenchymal injection is required to enable the planning and execution of trials developing therapies for demyelinating, degenerative, and traumatic spinal cord disease. Currently, there is no clear understanding of the number of injections that the large mammalian spinal cord can tolerate without transient and permanent morbidity. Similarly, spacing of injections is likely to affect morbidity. Moreover, macroscopic (*e.g.*, ventilation-related or inadvertent patient movement) and microscopic (*i.e.*, oscillation with both ventilation and cardiac pulse) spinal cord movements pose risks to the spinal cord during injection. An understanding of the threshold for morbidity in a large animal model will aid dosing calculations for all spinal cord therapy programs. Our translational spinal cord transplantation laboratory is available to help preclinical development programs of all teams currently designing trials for spinal cord application.

Disclosures

Dr. Boulis is the inventor of devices to enable safe and accurate injection of the human spinal cord. Neuralstem, Inc. has purchased an exclusive license to this technology. Dr. Boulis received an invento's share of this fee, and has the rights to royalty payments for distribution of this technology. Other authors have nothing to disclose.

Acknowledgements

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A QR Code is a matrix barcode readable by QR scanners, mobile phones with cameras, and smartphones. **The QR Code above links to Supplemental Digital Content from this article.**

Preclinical Validation of Multilevel Intraparenchymal Stem Cell Therapy in the Porcine Spinal Cord

BACKGROUND: Although multiple clinical trials are currently testing different stem cell therapies as treatment alternatives for many neurodegenerative diseases and spinal cord injury, the optimal injection parameters have not yet been defined.

OBJECTIVE: To test the spinal cord's tolerance to increasing volumes and numbers of stem cell injections in the pig.

METHODS: Twenty-seven female Göttingen minipigs received human neural progenitor cell injections using a stereotactic platform device. Cell transplantation in groups 1 to 5 (5-7 pigs in each) was undertaken with the intent of assessing the safety of an injection volume escalation (10, 25, and 50 μL) and an injection number escalation (20, 30, and 40 injections). Motor function and general morbidity were assessed for 21 days. Full necropsy was performed; spinal cords were analyzed for graft survival and microscopic tissue damage.

RESULTS: No mortality or permanent surgical complications were observed during the 21-day study period. All animals returned to preoperative baseline within 14 days, showing complete motor function recovery. The histological analysis showed that there was no significant decrease in neuronal density between groups, and cell engraftment ranged from 12% to 31% depending on the injection paradigm. However, tissue damage was identified when injecting large volumes into the spinal cord (50 μL).

CONCLUSION: This series supports the functional safety of various injection volumes and numbers in the spinal cord and gives critical insight into important safety thresholds. These results are relevant to all translational programs delivering cell therapeutics to the spinal cord.

KEY WORDS: Cell therapy, Neurodegenerative, Preclinical, Safety, Spinal cord, Tolerance, Transplantation

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The incomplete understanding of the underlying pathogenesis and the lack of an effective therapy for many neurodegenerative diseases affecting the spinal cord provide a unique atmosphere for the discovery of alternative therapeutics. Data on the pathogenesis of these diseases support both genetic and epigenetic causes.[1-3] Additionally, toxins, inflammation, excitotoxicity, and other causes have also been invoked.[4-9]

ABBREVIATIONS: hNPC, human fetal cortex–derived neural progenitor cell; **IM,** intramuscularly; **IV,** intravenously

Supplemental digital content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal's Web site (www.neurosurgery-online.com).

Development of cell therapies as treatment alternatives in conditions such as cancer and stroke has served as the scientific basis for the development of novel therapeutic strategies that could potentially be effective for various neurological diseases and/or for traumatic spinal cord injury.[10,11] In the past decade or so, neurons and other glial cells have been successfully generated from various types of stem cells.[12,13] Delivery of these cells to the spinal cord has been extensively studied, and, although highly invasive, intraparenchymal delivery is the most straightforward delivery method and is achieved by directly injecting the spinal cord using a cannula.[14,15] Stem cells and derived cells can be used to

replace diseased neurons, motor neurons, and/or glial cells.[16] These cells can be engineered to secrete cytokines and growth factors that promote neuroprotection and stimulate cell regeneration.[17] Furthermore, stem cells can be used to decrease inflammation through modulation of the local and systemic immune response.[18,19] Accumulated data from many preclinical studies support the use of cell replacement therapy as an effective alternative treatment for neurodegenerative diseases and spinal cord injury.[20-25] Although multiple clinical trials are currently under way using this approach to treat amyotrophic lateral sclerosis, multiple sclerosis, and spinal cord injury, the optimal injection parameters have not yet been defined.[26] The appropriate strategy for injections is dependent on an understanding of the tolerance of the spinal cord to multiple injection parameters. The purpose of this study was to test the spinal cord's tolerance to increasing volumes and total number of injections. We hypothesized that permanent neurological deficits would occur at or below 50 μL/graft and at or below 40 injections/procedure.

METHODS

The following section is an outline of the Methods; detailed methods are available (see **Methods**, **Supplemental Digital Content 1**, http://links.lww.com/NEU/A745).

Ethics Statement

This study was conducted at the University's Division of Animal Resources, following protocol approval by the Institutional Animal Care and Use Committee.

Study Design

The study design is summarized the in Table. Briefly, 27 healthy female Göttingen minipigs (Marshall BioResources, North Rose, New York), weighing 12 to 18 kg and divided into 5 groups, underwent multiple bilateral injections of cells into the spinal cord. Cell transplantation in groups 1 to 5 was undertaken with the intent to assess the safety of an injection volume escalation (10, 25, and 50 μL) and an injection number escalation (20, 30, and 40 injections).

TABLE. Experimental Design[a]

	Injection		
	Volume, μL	No	No. per Group
Group 1[b]	10	20 bilateral	7
Group 2	25	20 bilateral	5
Group 3	50	20 bilateral	5
Group 4	10	30 bilateral	5
Group 5	10	40 bilateral	4[c]

[a]A total of 27 healthy female Göttingen minipigs, ages 6 to 9 months and weight range 12 to 18 kg, were randomly assigned to 1 of 5 groups.
[b]Control group.
[c]One animal excluded from this group due to severe tissue damage while processing.

Cells Used for Transplantation

Dr Svendsen's laboratory at the Regenerative Medicine Institute at Cedars-Sinai Medical Center provided the human fetal cortex–derived neural progenitor cells (hNPCs) used in this study. The hNPCs, cultured as free-floating neurospheres, were received between passages 25 and 35.[27-29] Before transplantation, the neurospheres were dissociated to single cells, suspended in transplantation medium with a concentration of 10000 cells/μL, and maintained on ice.[30] Cell viability and concentration were calculated using a hemocytometer and trypan blue reagent. The minimum accepted viability percentage used was >80%.

Multilevel Laminectomy and Cell Transplantation

All animals were fasted for 12 hours before being anesthetized with ketamine (35 mg/kg intramuscularly [IM]), acepromazine (1.1 mg/kg, IM), atropine (0.02 mg/kg, IM); anesthesia was maintained during the procedure with isoflurane (1.5%-2.5%, inhaled) mixed with oxygen. After anesthesia, the pigs were placed in a prone position with appropriate draping of the operative area. A 10- to 15-cm incision was made over the cervical spine, and a multilevel laminectomy between levels 3 and 6 was performed over the cervical spinal cord. After laminectomy, the microinjection platform (Figure 1), described previously,[15] was mounted, allowing the device to span the laminectomy. A 2- to 4-cm incision was made in the dura mater, allowing exposure of the spinal cord. The dura mater was then tacked away using 4-0 Nurolon sutures (Ethicon, Inc, Somerville, New Jersey). At this point, the microinjection device was placed and adjusted. Targeting of the area of interest within the spinal cord was achieved as described in Figure 2. For each injection, a custom infusion cannula with a 29-gauge diameter was used, and the appropriate volume of cell suspension was infused by a microprocessor-controlled syringe pump (Microinjector; Tritech Research Inc, Los Angeles, California) at a rate of 5 μL/min.[15] The needle was left in place for an additional 1 minute to prevent cell reflux. After needle removal, the injection apparatus was relocated to the next target site using a spacing of 4 mm. This process was repeated as proposed in each volume and number escalation cohort. Once all injections were performed, the injection apparatus was removed, and the incisions were closed in 4 layers using the same technique reported previously.[31]

Postoperative Management

A standard immunosuppressive regimen of tacrolimus (Prograf; Astellas Pharma US Inc, Deerfield, Illinois) was used. Starting on the day of surgery, a dose of 0.0025 mg/kg, twice daily, was administered intravenously (IV) until euthanasia. Cefazolin (500 mg/day, IV) was administered starting on the day of surgery and maintained until postoperative day 10. Methylprednisolone (Solumedrol; McKesson Corporation, San Francisco, California) was administered as a bolus (125 mg IV) right after opening the dural sac. A fentanyl patch (75 μg) was placed for postoperative analgesia for 3 days.

Behavioral Assessment

Behavioral assessment of motor function was performed at baseline, daily during the 7 initial postoperative days, and then once weekly until euthanasia. Sensory evaluation took place in the form of a tactile stimulus to the interdigital space. All 4 limbs were assessed. Gait and motor function was assessed according to the Tarlov scale.[32] This scale provides objective criteria by which to evaluate the ability to ambulate as a surrogate measure of motor function. The scoring is as follows: 0, no voluntary limb function; 1, only perceptible joint movement; 2, active movement but unable to stand; 3, able to stand but unable to walk; 4 complete normal motor function.

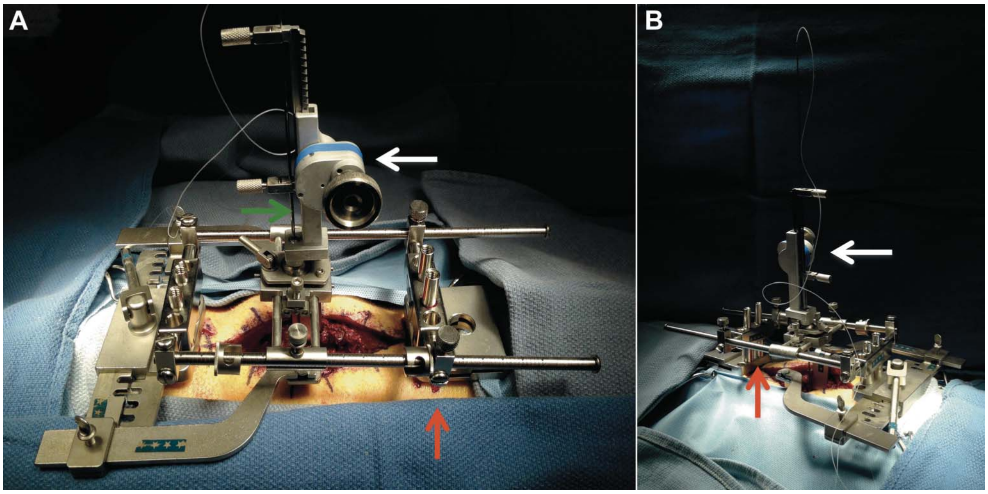


FIGURE 1. *Transplantation system. The stereotactic platform and injection device that were used for the intraparenchymal stem cell injections are shown. Lateral (**A**) and oblique (**B**) views. The solid red arrow indicates the stereotactic platform used to guide the injection device, the solid white arrow shows the injection device that holds the injection cannula, and the solid green arrow shows the 29-gauge custom-made cannula used for cell delivery.*

Euthanasia and Perfusion

At endpoints, animals were sedated with ketamine (35 mg/kg, IM), and acepromazine (0.8 mg/kg, IM). After sedation, 10000 units/mL of heparin sodium and euthasol (1 mL/10 lb) were administered IV while the heart was still beating. Transcardiac perfusion with a 0.9% sodium chloride solution followed by a 4% paraformaldehyde solution was then performed to improve the quality of the tissue for immunohistochemistry. A peristaltic pump (Masterflex Console Drive pump model 71-1420) was

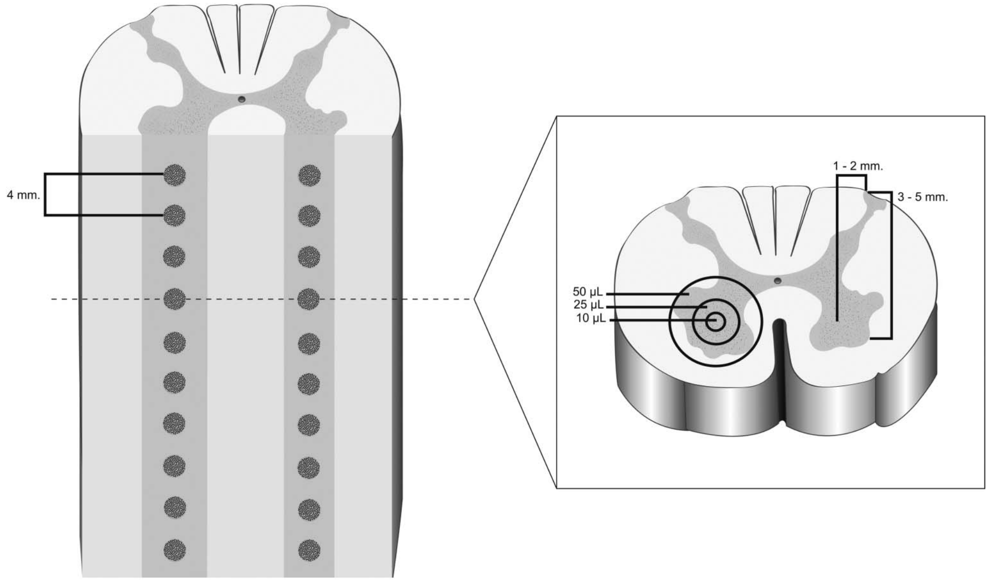


FIGURE 2. *Transplantation strategy. The ideal transplantation strategy using 10 µL to perform a total of 20 intraparenchymal spinal cord injections with 4-mm spacing. Targeting of the ventral horn was achieved using a 4-mm cannula and choosing the entry point 1 to 2 mm medial to the dorsal root entry zone. The coronal and sagittal angles are adjusted to ensure a trajectory orthogonal to the surface of the spinal cord. Additionally, this figure shows an approximate representation of the area of tissue that the different injection volumes would occupy with respect to the ventral horn of the gray matter.*

used for perfusions. Spinal cords were harvested and transferred to 30% sucrose in phosphate-buffered saline for 24 to 48 hours. Tissue was then frozen and stored in cryoprotectant.

Histology

Frozen coronal and transverse spinal cord sections (40-50 μm thick) were cut using a cryostat (Leica CM 1950; Leica Microsystems, Nussloch, Germany). Free-floating sections were stained with human nuclear antigen antibody (HuNu, catalog number MAB1281; Millipore Corporation, Billerica, Massachusetts) and counterstained with cresyl violet, mounted on slides, and coverslipped. Selected sections were stained for myelin with Luxol fast blue–periodic acid Schiff using a standard protocol. All images were captured with a digital DS-Qi1 high sensitivity Cooled CCD camera using a Nikon E400 microscope supplied with NIS-Elements imaging software (Nikon Instruments, Inc.).

Stereology Protocol

A uniform random sampling method was used to select a representative sample of the transplanted area for analysis (total distance between sections of 300 μm). A combination of the Cavalieri principle and the optical dissector was applied to the neuron and grafted cell counting.[33,34] A microscope (Leica DM2500) with a motorized x-y stage, an electronic microcator (Applied Scientific Instrumentation, Eugene, Oregon), and the PC software (Stereologer; SRC) were used for stereological analysis. All neurons and grafted cells that came into focus within the dissector height (20 μm) were counted, provided that they did not touch any of the exclusion lines and fell in the inclusion lines. The sections were analyzed using a 5× objective and 60× oil-immersion objective (final magnification, 2000×). The person performing the analysis of the spinal cords was blinded to the experimental design throughout the process.

Statistical Analysis

Descriptive statistics were used to report the outcome measures, and 1-way analysis of variance was used to test for statistically significant differences between groups. Tukey's post hoc comparisons were used to interpret the 1-way analysis of variance results. Significant differences were reported using a 0.05 α level.

RESULTS

Twenty-seven female Göttingen minipigs weighing12 to 18 kg received cervical intraspinal hNPC injections with a concentration of 10000 cells/μL. Different injection volumes (10, 25, and 50 μL) and total number of injections (20, 30, and 40 bilateral) were used for the transplantation technique (Table) in each 1 of the 5 groups (5-7 in each group).

Postoperative Behavioral Outcomes

Figure 3 shows both pre- and postoperative neurological outcomes for the volume escalation cohort (groups 1-3) and for the number escalation cohort (groups 1, 4, and 5). In all pigs assessed in groups 1 to 5, baseline sensory and motor function was regained by postoperative day 14 and maintained at postoperative day 21 when they were euthanized. One animal in each of groups 2 and 4 showed a slower trend towards recovery when compared with the rest of the group. Despite increasing injection volumes and total number, no statistically significant difference (F = 1.01, P = .42) was observed for the average time back to baseline among all groups. This indicates that neither increases in microinjection volumes nor total numbers were associated with the development of postoperative or permanent functional neurological sequelae.

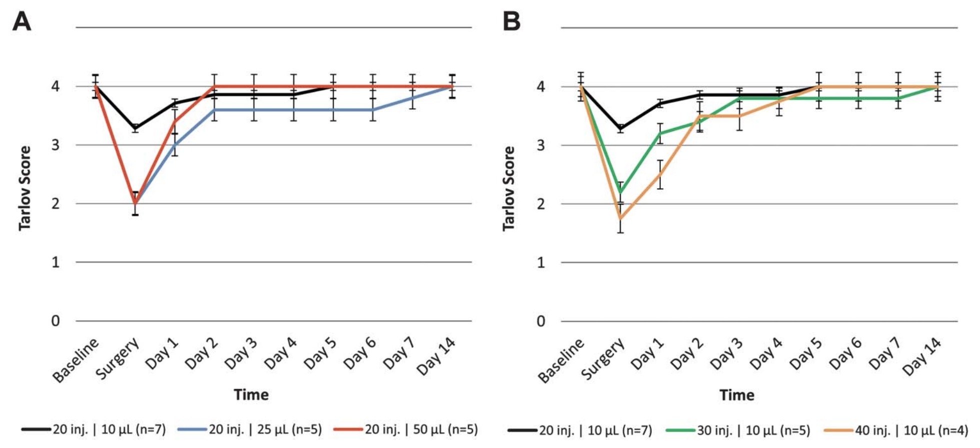


FIGURE 3. *Postoperative motor function assessments of experimental groups using Tarlov score. This figure shows the postoperative motor function assessment (Tarlov score) for the volume and number escalation cohorts.* **A**, *all animals in the volume escalation cohort came back to baseline at postoperative day 14 regardless of the injection volume.* **B**, *all animals in the number escalation cohort came back to baseline on postoperative day 14 regardless of the injection number. However, in both cohorts, one of the groups (20 inj/10 μL) appears to have better immediate postoperative morbidity compared with the rest of the groups. inj, injection.*

Postoperative Histological Outcomes

Descriptive statistics for the number of neurons per cubic millimeter and the number of damaged injection sites (primary measure of microscopic tissue damage) are shown on Figure 4. A damaged injection site was defined when >50% of the graft area was missing from the histological section (see **Figure**, **Supplemental Digital Content 2**, http://links.lww.com/NEU/A746). Engraftment percentage and total number of grafted cells are also reported.

Volume Escalation Outcomes

The stereological analysis of the volume escalation cohort showed that there was no statistically significant difference (F = 1.78, P = .21) in neuronal density at increasing volumes from 10 to 50 µL in groups 1 to 3. The histological analysis (Figures 5 and 6) showed a statistically significant (F = 12.01, $P \leq$ .001) difference between increasing volumes and damaged injection sites across groups 1 to 3. The pairwise comparison done using Tukey's post hoc adjustment method showed a significant difference in damaged injection sites between groups 1 and 3 ($P \leq$ .001) as well as between groups 2 and 3 (P = .04). In contrast, there was no significant difference in damaged injection sites between groups 1 and 2 (P = .16). These results indicate that there was no significant loss in neuronal density when increasing the injection volume. Although there was a significant increase in microscopic tissue damage at higher volumes, the fast blue–periodic acid Schiff stain did not show demyelination after transplantation using high injection volumes (see **Figure**, **Supplemental Digital Content 3**, http://links.lww.com/NEU/A747). Additionally, the engraftment percentage remained between 12% and 21%, depending on the injection volume, and group 3 (50 µL) showed the highest mean for number of engrafted cells (670832 hNPCs).

Two additional animals received twenty 75-µL injections with the intent of qualitatively assessing the presence of reflux and quantitatively assessing cell survival at high volumes. Both animals regained baseline motor function after 14 days. Harvested cords for these animals were sectioned in the axial and coronal planes to address both questions. Very few grafts showed mild to moderate reflux; however, most of the grafts analyzed in the axial plane showed no reflux. The stereological analysis of 16 cell grafts yielded 1607295 grafted cells, which corresponds to an 11% engraftment.

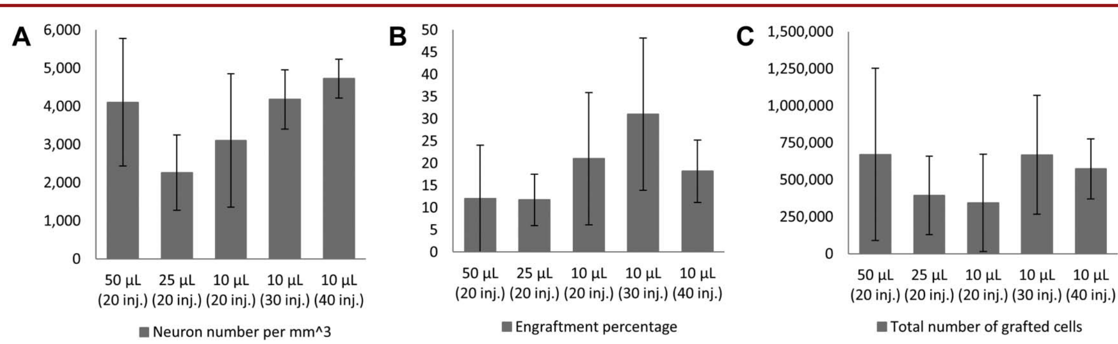
Number Escalation Outcomes

The stereological analysis of the number escalation cohort shows that there was no statistically significant difference (F = 2.27, P = .14) in neuronal density at increasing numbers of injections from 20 to 40 total across groups 1, 4, and 5. Furthermore, the histological analysis showed that there was no statistically significant (F = 3.50, P = .06) difference between increasing number of injections and damaged injection sites across groups 1, 4, and 5.

These results indicate that there was no significant difference in neuronal density, and there was no significant increase in tissue damage as the number of injections increased. The engraftment percentage remained between 18% and 31% depending on the injection number, with group 4 (30 injections) being the highest of all the groups. Group 4 also showed the highest mean for number of engrafted cells (667972 hNPCs).

DISCUSSION

The design of the current study was aimed at defining dose-limiting toxicity for stem cell transplantation in terms of graft



Outcome measure	Volume escalation (20 injections) 50 µL	25 µL	10 µL	Number escalation (10 µL) 30 injections	40 injections
Neuron number per mm^3	4,101 ± 1,671	2,260 ± 999	3,101 ± 1,749	4,178 ± 773	4,724 ± 513
Number of damaged injection sites	2 ± 1	0.8 ± 0.84	0 ± 0	0.6 ± 0.55	0.25 ± 0.5
Time back to baseline Tarlov score (days)	1.40 ± 0.55	2.8 ± 2.95	1.14 ± 1.77	2.8 ± 3.03	3.25 ± 1.50
Engraftment percentage	12.01 ± 12.07	11.74 ± 5.82	21.04 ± 14.89	31.07 ± 17.14	18.2 ± 7
Total number of grafted cells	670,832 ± 581,591	393,822 ± 264,216	343,805 ± 328,064	667,972 ± 401,573	573,746.5 ± 202,435

* Note: values reported are mean ± standard deviation

FIGURE 4. *Summary statistics of the outcome measures. Descriptive statistics of the outcomes measures chosen for this study are shown. Data reported correspond to the mean ± SD.* **A**, *there is no difference in neuron number/mm^3 across different injection paradigms.* **B**, *the best engraftment percentage is achieved when using a thirty 10-µL injection paradigm.* **C**, *the total number of grafted cells for each injection paradigm.*

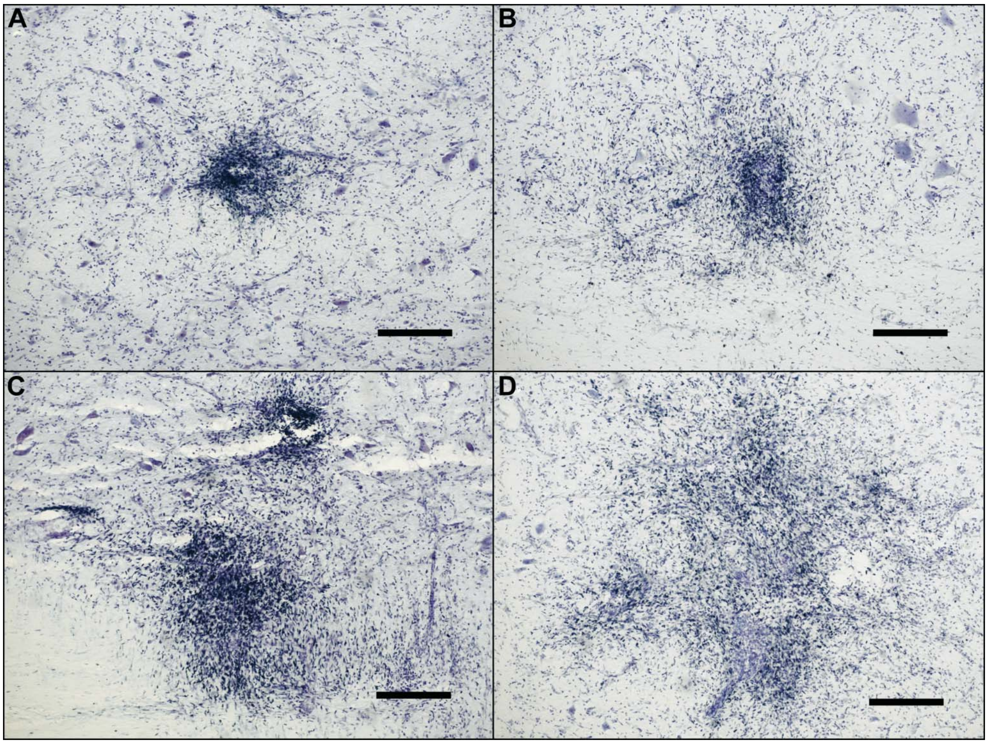


FIGURE 5. *Volume escalation histology. Coronal sections of a swine cervical spinal cord under bright field stained with human nuclear antibody for human fetal cortex–derived neural progenitor cells and counterstained with cresyl violet.* **A**, *10-μL graft;* **B**, *25-μL graft;* **C**, *50-μL graft;* **D**, *75-μL graft. Note the difference in distribution between the different injection volumes. The larger the volume, the further the transplanted cells spread from the graft's epicenter. Scale bar = 100 μm.*

volume and number of injections using a stereotactic delivery device.[14,15,35] The results of this study support the functional safety of various injection volumes and numbers in the spinal cord and gives critical insight into important safety thresholds based on histological findings.

Safety: Escalating Volume and Number of Intraparenchymal Spinal Cord Injections

We hypothesized that permanent neurological deficits would occur at or below 50 μL/graft and at or below 40 injections/procedure. This hypothesis was based on the assumption that

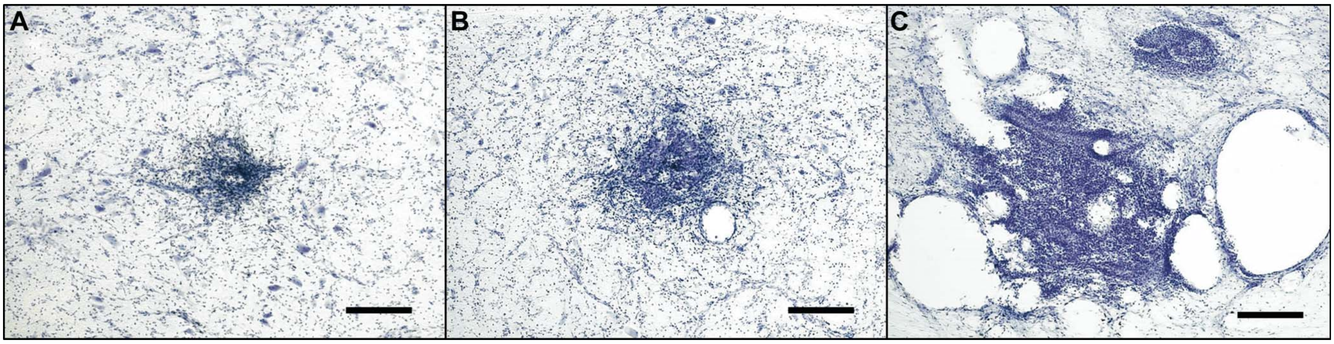


FIGURE 6. *Graft morphology. Coronal sections of a swine cervical spinal cord under bright field stained with human nuclear antibody for human fetal cortex–derived neural progenitor cells and counterstained with cresyl violet.* **A**, *a healthy 10-μL graft;* **B**, *a 50-μL graft with some infiltration;* **C**, *a 50-μL graft with significant infiltration and tissue damage. Scale bar = 100 μm.*

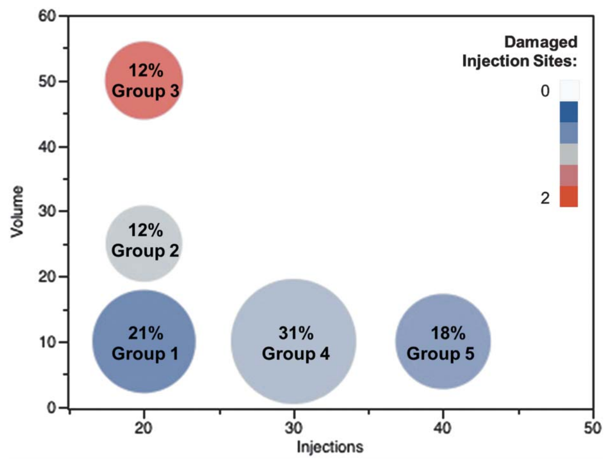


FIGURE 7. *Comparison of injection number and volume sized by engraftment percentage and colored by the number of damaged injection sites. This graph shows that as injection volume increases, the number of damaged injection sites increases and the engraftment percentage remains similar among groups (12%-21%). Additionally, it shows that as the number of injections increases, tissue damage stays relatively the same, but at 30 injections, the best engraftment percentage is achieved (31%). It is important to note that increased trauma results in increased local inflammatory and immune response; thus, this might be a potential explanation for why a greater engraftment percentage was observed with 30 injections compared with 40 injections.*

repeated injections would lead to an increasing probability of significant cord hemorrhages and that increasing volume would eventually create an associated increase in intraspinal cord pressure.[36] Using magnetic resonance images of the pig's cervical spinal cord, the total volume of the cord's transplanted area yielded 2081 $mm^3/\mu L$, and the ventral horns (target area) volume yielded approximately 211 $mm^3/\mu L$. Consequently, 20 injections of 10, 25, and 50 µL would occupy approximately 10%, 24%, and 48% of the total spinal cord volume, respectively, and our lowest injection volume would occupy almost all of our target area. To our surprise, the findings in this study proved that increasing the intraspinal injection volume to 50 µL (and perhaps 75 µL; n = 2) and the total injection number up to 40 result in no permanent neurological morbidity. All animals in the volume and number escalation groups returned to preoperative sensory and motor function within 14 days after the surgery was performed; however, transient morbidity should not be neglected. Transient decline of motor function was seen in all animals during the first postoperative week; this decline was less severe (Figure 3) in group 1 (20 bilateral 10-µL injections) when compared with all other groups. However, this difference might be confounded by the fact that the animals receiving higher volumes or a greater number of injections required a longer surgical procedure. In our initial clinical trials, humans do not seem to exhibit the same transient motor decline, but this difference may be attributed to cross-species differences in central pain processing.[37] Nonetheless, we believe that the spinal cord tolerance demonstrated in the current study will translate well to human surgery.

Previous research studies have looked at the safety and accuracy of delivering cell therapies to the spinal cord.[14,15,35] However, these studies did not include quantitative assessment of histological tissue damage as a measurement of safety. Thus, histology outcomes in this study were chosen to assess tissue damage at the cellular level that might be undermined by the limited sensitivity of the Tarlov scale.[38,39] Additionally, other histological outcomes such as engraftment percentage and total number of grafted cells were selected to assess which injection paradigm yielded greater cell survival.

The behavioral and histological outcomes analyzed in this work provide the means to define safety thresholds for intraspinal injection volume and number. Thus, the optimal injection paradigm should use an injection volume between 10 and 25 µL at a fixed concentration of 10000 cells/µL. Furthermore, the number of injections can be safely increased to a total of 40 when using a volume of 10 µL (Figure 7). These safety thresholds should be interpreted with caution when choosing an injection strategy for other applications or diseases. It is also important to account for other factors such as anatomic location, underlying disease, patient characteristics, cell type and concentration, delivery vehicle, and infusion rate.

In addition to the 27 animals in the study cohort, 2 experimental animals that received 20 (75-µL) injections were included with the intention of assessing injection reflux at high volumes. Of the total grafts analyzed in these animals, only mild to moderate reflux was noted along the cannula tract. This finding contradicts our initial assumption that this high volume of injection would cause some reflux. The floating cannula is designed to move with the cord, hence maintaining surface tension that we believe would resist reflux. However, the evaluation of reflux is limited by the fact that it was only assessed at 1 time point (postmortem, 21 days after transplantation) and could have occurred during injection.

Clinical Translation: From Bench to Bedside

The results provide critical insight toward answering the question of what the optimal injection paradigm is for cell therapies targeting the spinal cord across a variety of diseases. The chosen outcomes helped define important safety thresholds that can be used for the delivery of other types of therapeutics such as viral vectors or proteins.[40-42] Our current human trials have focused on segmental therapy of the C3-5 region of the spinal cord to preserve diaphragmatic function in the hope of prolonging tracheostomy-free survival in amyotrophic lateral sclerosis patients. The current work suggests that multiple smaller injections may be optimal, thus supporting the expansion of our segmental approach to transplantation of all the cervical segments innervating the upper extremities to

preserve arm as well as diaphragmatic function.[26,37,43] Looking into the future approaches using magnetic resonance guidance for targeting and delivery could provide minimal invasiveness and also real-time control of the delivery of cell therapeutics to the spinal cord.

Limitations

The limitations of this study include the small number per group and the limited sensitivity of the behavioral evaluations.

CONCLUSION

The results of this work demonstrate the functional safety of escalating microinjection volume and number in the cervical spinal cord of Göttingen minipigs. Histological outcomes served as additional measures that allowed us to better determine safety thresholds. The findings of this study are relevant to all translational programs currently attempting to deliver cellular therapeutics to the spinal cord.

Disclosures

Dr Boulis is the inventor of devices to enable safe and accurate injection of the human spinal cord. Neuralstem, Inc. has purchased an exclusive license to this technology. He also received an inventor's share of this fee and has the rights to royalty payments for distribution of this technology. The other authors have no personal, financial, or institutional interest in any of the drugs, materials, or devices described in this article.

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COMMENT

The authors present a well-written and important analysis in an evolving and growing field of treatment of cell-mediated spinal cord repair. They use the spinal cords of minipigs to evaluate the spinal cord's ability to tolerate increasing volume and number of injections. This is a very important concept in that currently there are several "stem cell" trials under way throughout the world. Unfortunately, there is no known optimal concentration, and much is being learned through the ongoing clinical trials. The study allows further definitions to set boundaries in future clinical trials to maximize clinical benefit and reduce potential neural injury.

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Neurosurgery

Understanding Cell Distribution After Direct Transplantation Into the Spinal Cord: A Tool to Calculate the Optimal Transplantation Volume

--Manuscript Draft--

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Abstract:	Introduction: Cell therapies represent a promising alternative treatment for neurodegenerative diseases of the spinal cord, and traumatic spinal cord injury. Cell migration, distribution, proliferation and differentiation are factors that greatly influence the therapeutic potential of cell therapies. Objective: This study analyzed the distribution patterns of human fetal-derived neural progenitor cells (hNPCs) transplanted to the spinal cord of healthy minipigs. Materials and Methods: Fifteen minipigs divided into 3 groups received twenty bilateral 10, 25, or 50-microL intraspinal injections of hNPCs at a concentration of 10,000 cells/microL. Following 21 days, animals were euthanized, perfused, and spinal cords were collected for immunohistochemistry. Cell grafts (n=5 per group) in both white matter (WM) and grey matter (GM) were quantitatively assessed in the three-dimensional and in the two-dimensional space to calculate the distance cells traveled in the rostro-caudal and transversal planes. Results: Cell grafts exhibited different distribution patterns in each anatomical compartment, regardless of injection volume. Cell grafts found in the WM travelled more in the rostro-caudal plane, whereas cell grafts found in the GM travelled similarly in the rostro-caudal and transversal planes. The total volume of GM occupied by the 25-microL grafts was significantly larger (p=0.02) when compared to the 10-microL grafts, but not significantly smaller when compared to the 50-microL grafts. These results suggest that 25 microL (10,000 cells/microL) is the optimal injection volume. Conclusion: Understanding the dynamics of different cell lines will allow neurosurgeons to maximize accurate delivery and effectiveness of cell therapeutics in the spinal cord.

Additional Information:	
Question	**Response**
Significance of the Work: Please include a brief statement summarizing the significance of the work and in particular how it differs from and advances existing literature.	The available body of literature provides mainly qualitative information on behavioral characteristics of a multitude of cell types after transplantation into the Central Nervous System. This data is generated based on a range of delivery methods that influence cell distribution/migration including variations in cell line, delivery site, injection volume, and cell concentration. Understanding cell dynamics after transplantation will allow neurosurgeons to ensure accurate delivery and maximize efficiency of cell therapeutics in the spinal cord. More importantly, by using the quantitative tools described to measure cell distribution in this study, neurosurgeons and scientists will be able to determine the optimal injection volume for a particular cell line.
Compliance with Research Reporting Guidelines: *Neurosurgery* endorses several reporting guidelines and requires authors to submit their research articles in accordance with the appropriate guideline statement(s) and checklist(s). Completed applicable checklists and flow diagrams must be included with submissions. Research articles that must be submitted according to the appropriate reporting guideline(s) include, but are not limited to: randomized trials, systematic reviews, meta-analyses of interventions, meta-analyses of observational studies, diagnostic accuracy studies, and observational epidemiological studies (eg, case series, cohort, case-control, and cross-sectional studies). Consult the EQUATOR Network, which maintains a useful, up-to-date list of guidelines as they are published, with links to articles and checklists: http://www.equator-network.org. Please confirm below that information is reported according to the relevant reporting guideline(s) and any required materials are included with the submission:	Not Applicable - Submission Does Not Report Research That Requires Adherence to Reporting Guideline(s)

STEM CELL DISTRIBUTION IN THE SWINE SPINAL CORD

Understanding Cell Distribution ion after Direct Transplantation into the Spinal Cord − A Tool to Calculate The Optimal Transplantation Volume.

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Disclaimers: Nicholas M. Boulis, MD is the inventor of devices to enable safe and accurate injection of the human spinal cord. Neuralstem, Inc. has purchased an exclusive license to this technology. Dr. Boulis received an inventor's share of this fee, and has the rights to royalty payments for distribution of this technology. Dr. Boulis also serves as a consultant for Neuralstem, Q Therapeutics, MRI Interventions, Agilis, Biomedica, and Voyager.

Acknowledgements: This work was funded by the United States Department of Defense Amyotrophic Lateral Sclerosis Research Program – Therapeutic Research Award; and supported in part by the NIH/FIC (D43TW01042), and the NIH/NCATS (UL1TR000454). We thank the Emory University Division of Animal Resources for veterinary care.

Understanding Cell Distribution After Direct Transplantation Into The Spinal Cord − A Tool To Calculate The Optimal Transplantation Volume.

ABSTRACT

Introduction: Cell therapies represent a promising alternative treatment for neurodegenerative diseases of the spinal cord, and traumatic spinal cord injury. Cell migration, distribution, proliferation and differentiation are factors that greatly influence the therapeutic potential of cell therapies.

Objective: This study analyzed the distribution patterns of human fetal-derived neural progenitor cells (hNPCs) transplanted to the spinal cord of healthy minipigs.

Materials and Methods: Fifteen minipigs divided into 3 groups received twenty bilateral 10, 25, or 50-microL intraspinal injections of hNPCs at a concentration of 10,000 cells/microL. Following 21 days, animals were euthanized, perfused, and spinal cords were collected for immunohistochemistry. Cell grafts (n=5 per group) in both white matter (WM) and grey matter (GM) were quantitatively assessed in the three-dimensional and in the two-dimensional space to calculate the distance cells traveled in the rostro-caudal and transversal planes.

Results: Cell grafts exhibited different distribution patterns in each anatomical compartment, regardless of injection volume. Cell grafts found in the WM travelled more in the rostro-caudal plane, whereas cell grafts found in the GM travelled similarly in the rostro-caudal and transversal planes. The total volume of GM occupied by the 25-microL grafts was significantly larger (p=0.02) when compared to the 10-microL grafts, but not significantly smaller when compared to the 50-microL grafts. These results suggest that 25 microL (10,000 cells/microL) is the optimal injection volume.

Conclusion: Understanding the dynamics of different cell lines will allow neurosurgeons to maximize accurate delivery and effectiveness of cell therapeutics in the spinal cord.

Keywords: Cell therapies, cell dynamics, injection volume, spinal cord, transplantation.

Short title: Stem cell dynamics in the swine spinal cord.

INTRODUCTION

Neurodegenerative diseases (NDs) and traumatic spinal cord injury (SCI) are conditions that present a major burden on the patient. Current research studies provide compelling evidence that cell therapies applied to the spinal cord ameliorate symptoms arising from SCI and some NDs[1-7]. These cell therapies attempt to either provide neuronal replacement and/or to decrease the toxicity of non-neuronal cells with the intention of enhancing the local microenvironment[8]. Despite the inherent differences of each approach they both try to prolong host cell survival, while maximizing transplanted cell distribution, migration to target site, and differentiation into mature neuronal or glial cells[6, 9].

Several observations regarding distinct stem cell distribution and/or migration patterns after transplantation into the spinal cord have been previously described[10-13]; however factors affecting these dynamics are inconsistent and are reported in a qualitative manner across studies[14, 15]. Thus, developing quantitative measures of cell distribution and/or migration could be extremely useful in order to fairly assess and compare the multitude of stem cell types that are being studied today. Data generated through the use of these measurements will allow us to better understand the inherent properties of different stem cells lines and to translate these properties to improve surgical designs thus enhancing the therapeutic potential of each lineage. The present study focused on quantitatively assessing distribution patterns of human fetal-derived neural progenitors (hNPCs) transplanted to the white matter (WM) and grey matter (GM) compartments of the spinal cord in healthy Göttingen minipigs using stereological volumetric calculations and Image J software[16, 17].

MATERIALS AND METHODS

The following section is a brief description of the Materials and Methods; full details are available in the Supplemental Digital Content 1.

Ethics Statement

This study was carried out following the guidelines of the Institutional Animal Care and Use Committee at the University's Division of Animal Resources.

Study Design

Fifteen healthy female Göttingen minipigs received twenty bilateral 10, 25, or 50 µL injections of hNPCs into the spinal cord (Table 1). Following 21 days, animals were euthanized, perfused, and the spinal cord was collected for immunohistochemistry. Cell grafts were quantitatively assessed in the three-dimensional space by stereological volumetric calculations and in the two-dimensional space using Image J to measure distance travelled from epicenter.

Cells Used for Transplantation

The Svendsen's lab provided the hNPCs used in the study. hNPCs were received as free-floating neurospheres between passages 25 and 35[18-20]. Prior to transplantation, neurospheres were dissociated into single cells, then suspended in transplantation medium at 10,000 cells/µL, and maintained on ice[21]. The minimum accepted cell viability was >80% as determined using a hemocytometer and Trypan blue reagent.

Multilevel Laminectomy and Cell Transplantation

All animals were fasted for ≥12 hours prior to being anesthetized with Ketamine (35 mg/kg, IM), Acepromazine (1.1 mg/kg, IM), Atropine (0.02 mg/kg, IM), and maintained

intraoperatively with Isoflurane (1.5-2.5%, Inhaled) diluted in oxygen. A multi-level laminectomy between cervical levels 3 to 6 was performed. As described in previous publications[22, 23], the microinjection apparatus was attached to four percutaneous posts allowing it to span the laminectomy. The spinal cord was then exposed; twenty bilateral 10, 25, or 50 µL injections of cell suspension were infused at a rate of 5 µL/ minute using a 29-gauge custom made cannula. Once all injections were performed, the injection apparatus was removed, the dura mater and the rest of the incision was closed using the same technique reported in previous publications[22, 23].

Postoperative Management and Euthanasia

Postoperative care was provided in the same manner as previously described[23], using a standard immunosuppressive regimen of Tacrolimus (Prograf – Astellas Pharma US Inc., Deerfield, IL). For euthanasia, animals were sedated with Ketamine (35 mg/kg, IM), and Acepromazine (0.8 mg/kg, IM) followed by a lethal dose of Pentobarbital (0.1 ml/lb) on postoperative day 21. Subjects were transcardially perfused with 0.9% NaCl followed by a 4% paraformaldehyde solution (PFA). Spinal cords were harvested, stored in 4% PFA for 24 hours at 4ºC, then transferred to 30% sucrose in phosphate-buffered saline (PBS) for 7 days. Tissue was flash frozen using 2-methylbutane and stored at -80ºC.

Tissue Histology

Coronal sections were cut at 40-50 µm, and stored as free-floating sections in cryoprotectant. Sections were washed in PBS and blocked with horse serum (Jackson Immuno-Research) for one hour. Primary antibodies directed against the human nuclear antigen (HuNu - Millipore, MAB1281) were incubated overnight at 4ºC, and stained with a secondary horse anti-mouse

IgG (Vector Labs). Tissue was incubated overnight with tertiary antibodies from the ABC kit (Vector Labs) and amplified with Peroxidase Substrate (SG-black SK-4700). A Cresyl Violet counter-stain was used to provide background. A similar staining protocol was used to detect transplanted cell apoptosis using a human-specific Caspase-3 antibody (CC-3, Cell Signal catalog #9661). Images were captured using a Nikon E400 microscope supplied with NIS-Elements imaging software (Nikon Instruments, Inc.).

Microscopic Analysis

Representative cell grafts of each injection volume cohort in both WM (n=5) and GM (n=5) were selected randomly for analysis. Cell grafts that showed significant inflammatory response, cell rejection or tissue damage were excluded from the analysis. For the two-dimensional distribution analysis the slide containing the cell graft epicenter (slide with greatest number of HuNu positive cells) was used. As shown in Figure 1, rostro-caudal and transverse migration was determined as the distance traveled by cells from the furthermost points on either side of the epicenter of the graft. Measurements were done using a length probe provided in the image processing software, Image J[17].

Stereology was used to estimate three-dimensional graft distribution. A uniform-random sampling technique was used to select a representative sample (6-8 sections) for analysis of the cell grafts in the WM (n=5) and GM (n=5), with a total distance between sections of 300 μm. The Cavalieri method was used to estimate three-dimensional volume of each cell graft using a 5X objective[24, 25]. A microscope (Leica DM2500) with a motorized x–y stage, an electronic microcator (Applied Scientific Instrumentation), and the PC software Stereologer™ were used for the analysis.

Statistical Analysis

Descriptive statistics of the chosen microscopic outcomes are presented as mean ± standard deviation. Differences between groups were assessed using one-way ANOVAs with Tukey's post-hoc pairwise comparison using an alpha of 0.05.

RESULTS

Distribution Analysis

A marked distinction between distribution patterns in the WM and GM was apparent in both rostro-caudal and transverse planes, regardless of injection volume (Figure 2). Cell grafts in the GM distribute similarly in all directions, thus resulting in a spherical graft, while cell grafts in the WM had an elongated shape due to preferential distribution along the rostro-caudal axis. Quantitative analysis of cell distribution in WM and GM further supported this site-specific behavior, and showed significant differences in both rostro-caudal and transverse planes throughout different injection volumes (Figure 3).

To understand how injection volume influences distribution in the rostro-caudal and transverse planes, we compared mean distance from graft epicenter between the 10, 25 and 50 µL groups. In the WM, cells traveled significantly farther in the transverse plane as injection volume escalated ($p=0.03$). However, cells traveled comparable distances longitudinally despite a five-fold increase in volume. In the GM, increasing injection volume resulted in greater distribution in both planes, but the difference was not statistically significant (Figure 3).

Volumetric Analysis

Stereological analysis was used to quantify the three dimensional area that cell grafts occupied as injection volume increased. In the WM, volume of cell grafts increased significantly as injection volume increased ($p=0.03$). Similarly, GM grafts were larger in volume as the injection volume escalated ($p=0.01$); however, this difference was not significant between 25 and 50 μL groups (Figure 4).

Grey-White Matter Junction

Some cell grafts that were not included for this analysis demonstrate characteristic behavior when approaching the grey-white matter junction (Figure 5). Cells transplanted into the GM did not cross the junction into the WM, and vice versa for cells transplanted in the WM. Additionally; cells appear to distribute along the junction when transplanted close to this site.

DISCUSSION

The available body of literature provides mainly qualitative information on behavioral characteristics of a multitude of cell types after transplantation into the CNS[12, 13, 26, 27]. In this study cell grafts exhibited different distribution patterns in each anatomical compartment, regardless of injection volume. Furthermore, the results suggest that 25 μL is the optimal injection volume for this cell line.

Cell Distribution in White versus Grey Matter

hNPCs displayed site-specific distribution when transplanted into the WM and GM, this probably due to the difference in cell density and/or cytoarchitecture between both anatomical compartments. Statistically significant differences were observed along the rostro-caudal and transverse axis in both anatomical compartments of the spinal cord. Cells transplanted to the

WM travel significantly farther rostral and caudally compared to cells transplanted to the GM. Conversely, cells transplanted to the GM distributed significantly more transversally when compared to WM. As shown in Figure 2, these differences between compartments resulted in characteristically distinct cell graft shapes. In the WM, increasing injection volume appeared to have a statistically significant impact on cell graft width. To our surprise, increasing injection volume five-fold showed no significant increase on rostro-caudal distribution, countering our initial thought that by increasing injection volume cells would travel further along the WM axon bundles. However, a trend of preferential distribution/migration of stem cells along WM tracts has been previously reported[10, 13]. Therefore, increasing injection volume in the WM may not produce much difference if maximizing area coverage is the main therapeutic objective.

Cell Graft Volume in White versus Grey Matter

Figure 4 shows a positive correlation between injection volume and cell graft volume in the WM. Conversely in the GM, the estimated cell graft volume was significantly larger when increasing the injection volume from 10 to 25 µL, but not from 25 to 50 µL. This data suggests that injecting volumes above 25 µL might result in greater cell death, although the limited amount of the apoptotic marker Caspase-3 found in the cell grafts might suggest otherwise (Supplementary Figure 1). Furthermore, the size of the grafts produced when injecting volumes above 25µL were marginally larger; thereby not justifying the risk associated with injecting higher volumes as the GM has been proven to be more susceptible to strain failure than WM at lower pressures[28].

Clinical Applications

The variance seen in cell distribution among spinal cord compartments can be exploited by tuning delivery techniques during the surgical process. For example, researchers are currently

using stem cells engineered to deliver trophic support to the spinal cord in ALS animal models[19, 29]. Coupling this paradigm with delivery to the WM using a cell line that distributes robustly could maximize coverage along the spinal cord, thus maximizing the therapeutic effect[19, 30, 31]. Conversely, cell replacement therapies (i.e. for SCI) would most likely target the ventral horns of the grey matter. In each scenario, surgical precision however will be highly important to ensure delivery to the appropriate anatomical compartment. Research studies on SCI currently aim to transplant cells to the grey matter above and below the site of injury. However, based on the present study and other research, this compartment appears to impose resistance to cell distribution, thus possibly having an impact in migration and proliferation. Cells used the present study travelled less than half a millimeter at optimal injection volume over three weeks time when transplanted into the GM. However, one must keep in mind that cells behave differently when transplanted into the injured spinal cord[32-34]. Furthermore, some stem cell lines have shown to be unable to cross the grey-white matter junction. Thus, in some disease states, attempts can be made to supplement GM grafts by injecting into the WM to enhance trophic support[35].

In the future, cell-labeling and MRI-guided surgical delivery will provide ease of precision and cell-tracking in real-time. Until these tools are available, it is important to quantitatively characterize the behavior of stem cell lines in a way to fairly assess them across studies.

CONCLUSION

Understanding cell dynamics after transplantation will allow neurosurgeons to determine optimal injection parameters in order to ensure accurate delivery and maximize efficiency of cell therapeutics in the spinal cord.

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FIGURE LEGENDS

Figure 1. Description of the method used to measure cell distribution with Image J software. Distribution was measured in the grey (A) and white (B) matter using a length probe placed through the graft epicenter from the furthest points where HuNu positive cells resided in the rostro-caudal (red arrows) and transverse (green arrows) planes. Scale bars = 1mm.

Figure 2. Comparison of graft morphology. hNPC grafts transplanted into the grey matter of the cervical spinal cord display a spherical pattern following both maximal (A) and minimal (B) distribution. hNPC grafts into the white matter display an elongated pattern following maximal (C) and minimal (D) distribution. Scale bars = 1mm.

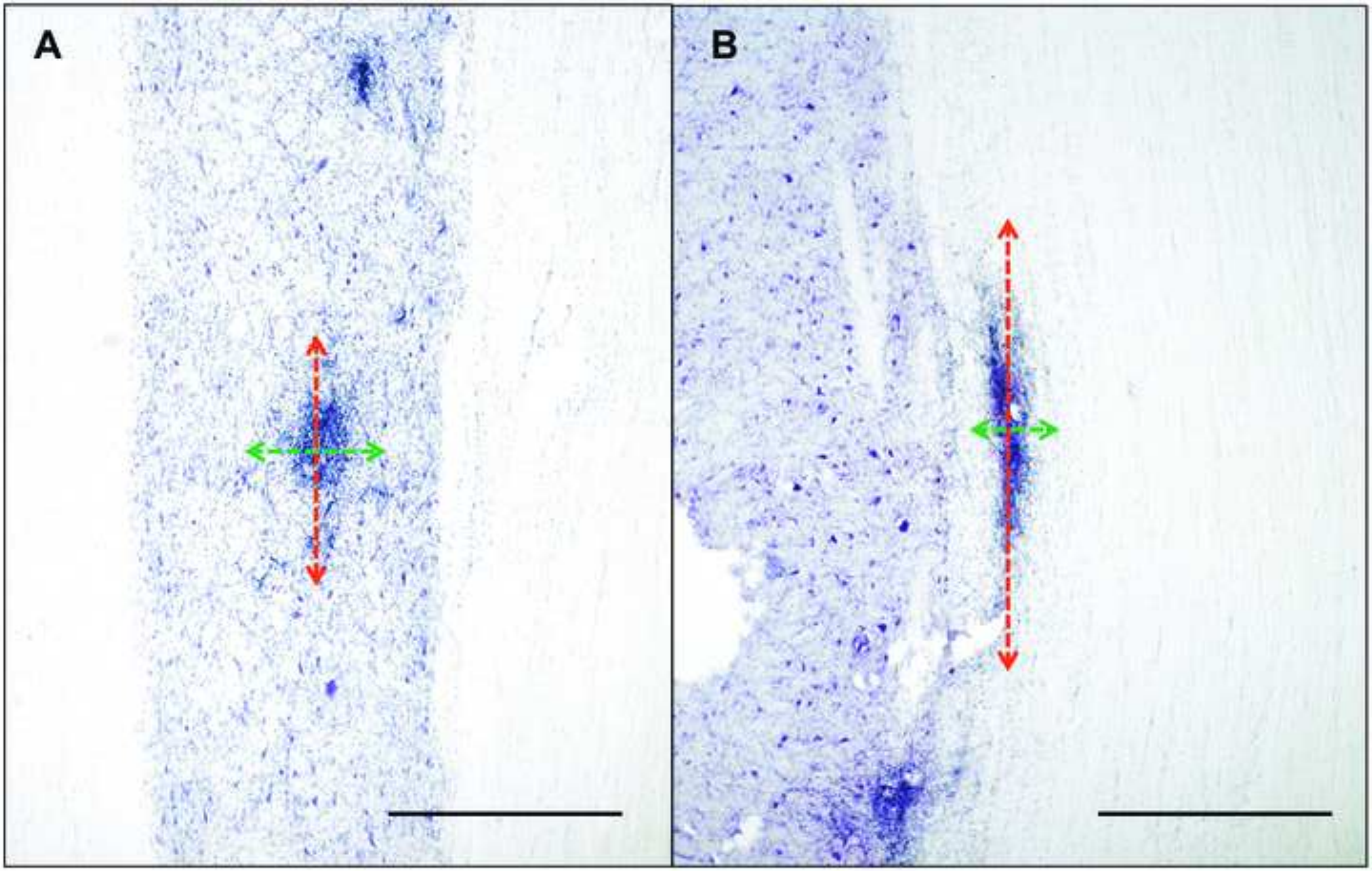
Figure 3. Rostro-caudal and transverse cell distribution in white versus grey matter. Cell distribution in the grey and white matter increases with increased injection volume, but the difference was only significant on the transverse plane in the white matter. However, note that rostro-caudal cell distribution increases significantly when cells were transplanted to the white versus grey matter regardless of the injection volume. Data is expressed as the mean ± standard deviation (*Alpha = 0.05).

Figure 4. Volume occupied by cell grafts using different injection volumes. Estimated cell graft volume in the white matter was significantly larger when comparing the 10 and 50 µL groups (p=0.01; post-hoc t-test). In the grey matter, grafts were significantly larger when comparing the 10 vs. 25 µL (p=0.02), and not significantly larger when comparing the 25 vs. 50 µL (p=0.57) groups. Data is expressed as the mean ± standard deviation (*Alpha = 0.05).

Figure 5. Graft morphology at the grey-white matter junction in the cervical spinal cord. hNPC grafts in the white (A) and grey matter (B and C) exhibit cell distribution along, but not

across the grey-white matter junction. A possible explanation to this phenomenon might be the difference in cell density and/or cytoarchitecture between both anatomical compartments. However, intrinsic characteristics of the cell line might have also played a role. The blue arrows indicate the grey-white matter junction, the red arrows indicate the white matter, and the green arrows indicate the grey matter. Scale bars = 1mm.

Supplementary Figure 1. Human-specific apoptotic marker Caspase-3 histology. Different injection volumes were stained for cleaved Caspase-3. (A) 10 μL cell graft, (B) 25 μL cell graft, and (C) 50 μL cell graft, all with insets taken in the graft center. Note the minimal amount of black halos that can be appreciated around the nuclei of some of the cells in each cell graft. Most cell death is expected to occur during cell transplantation up to 2 weeks posterior to this. Thus, it is important to highlight that these animals were sacrificed 21 days post-transplantation, so this histology might be a limited representation of cell death occurring due to high injection volumes. Scale bars = 1 mm.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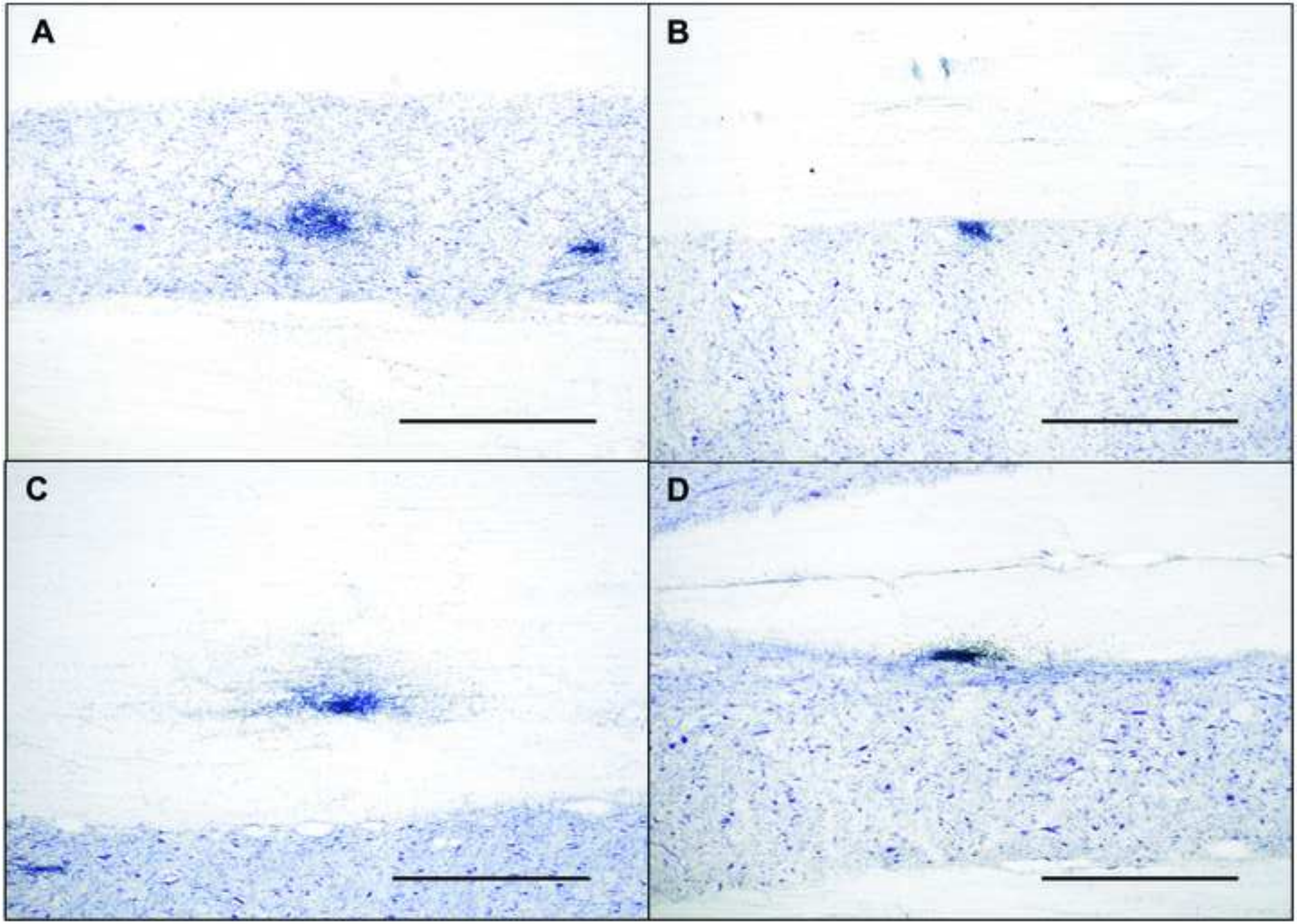
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Figure

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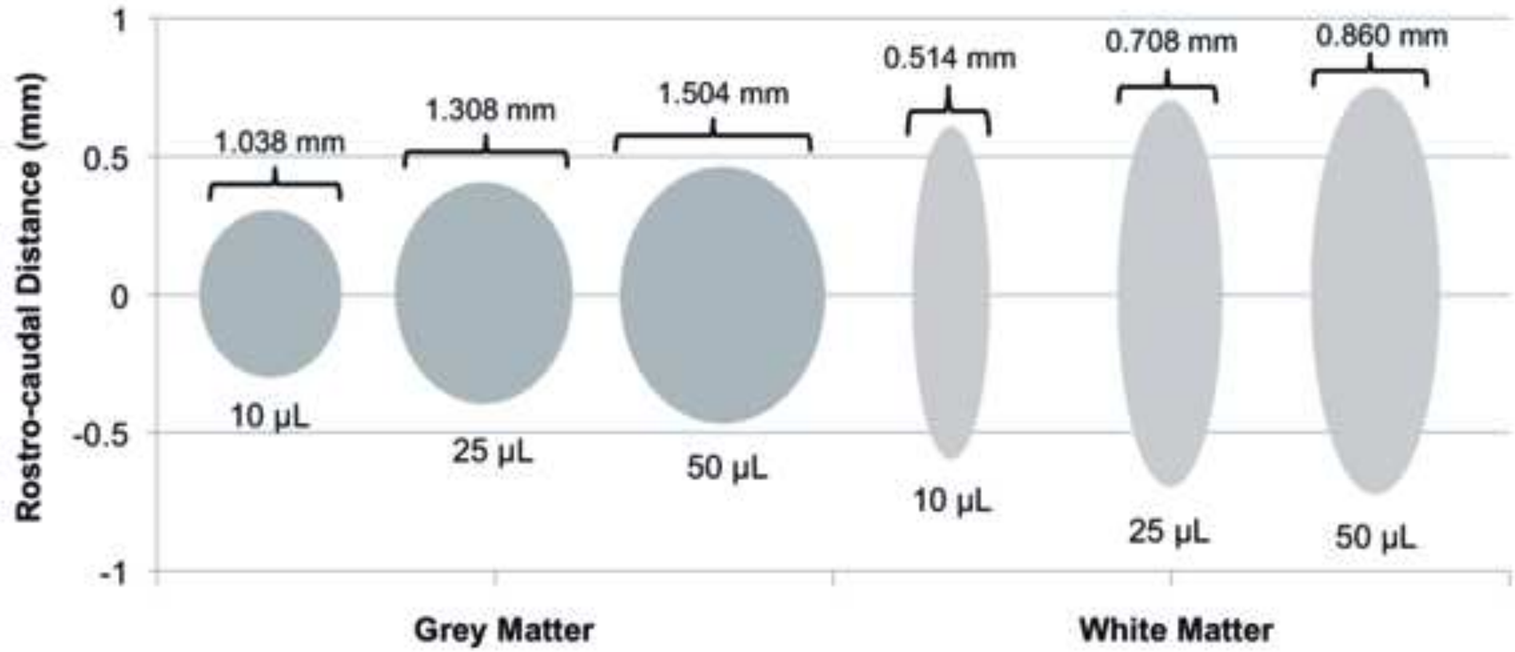


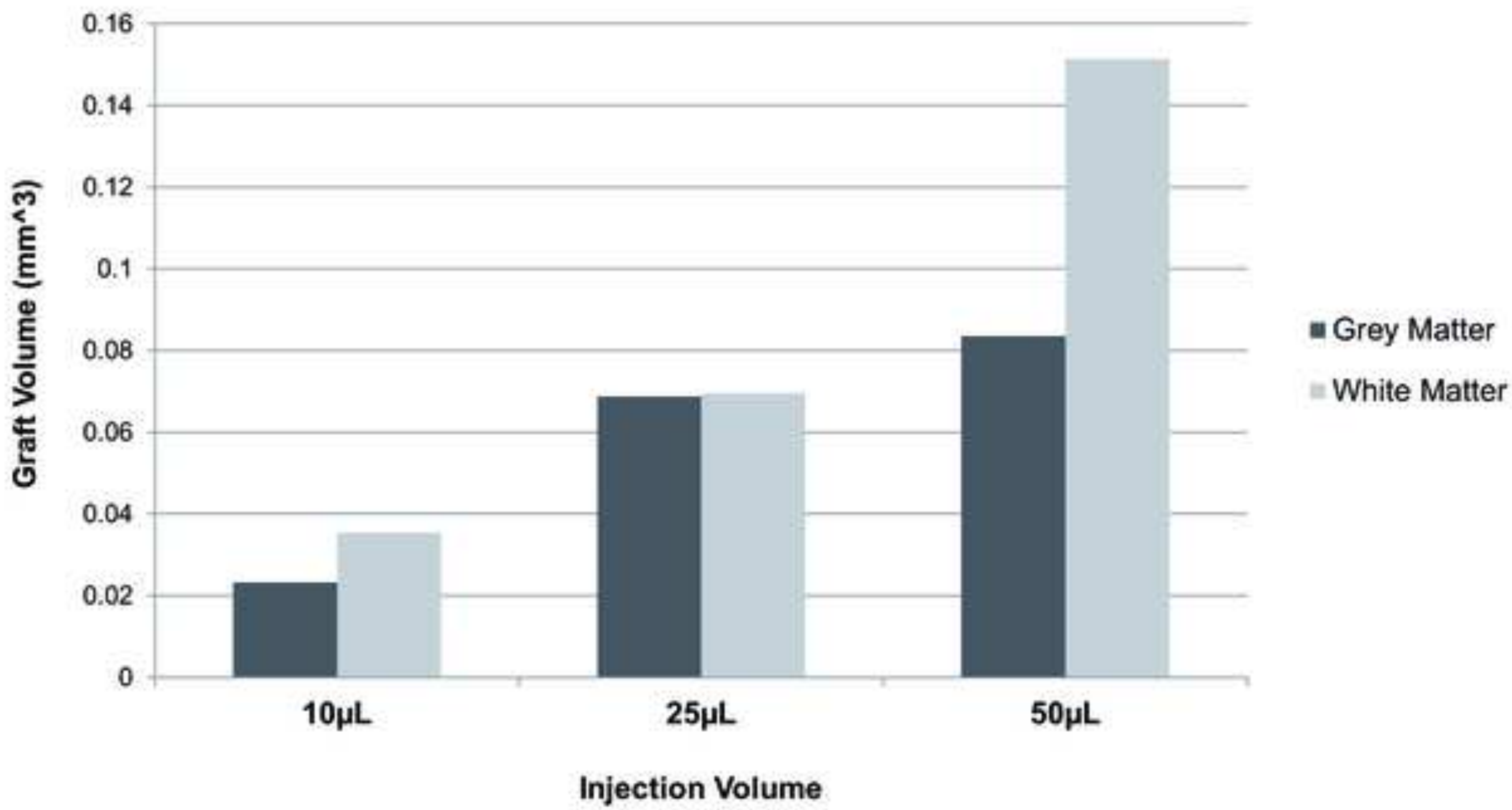
Rostro-caudal distribution from epicenter

	Injection Volume			
Injection Location	**10 μL (n=5)**	**25 μL (n=5)**	**50 μL (n=5)**	**ANOVA**
Grey Matter	0.619 ± 0.195 mm	0.818± 0.375 mm	0.946 ± 0.123 mm	0.16
White Matter	1.116 ± 0.412 mm	1.304 ± 0.161 mm	1.374 ± 0.243 mm	0.38
T-test	0.04*	0.03*	<0.01*	

Transverse distribution from epicenter

	Injection Volume			
Injection Location	**10 μL (n=5)**	**25 μL (n=5)**	**50 μL (n=5)**	**ANOVA**
Grey Matter	0.519 ± 0.182 mm	0.654 ± 0.333 mm	0.752 ± 0.244 mm	0.39
White Matter	0.257 ± 0.090 mm	0.345 ± 0.072 mm	0.430 ± 0.102 mm	0.03§
T-test	0.02*	0.08	0.03*	

§ Post-hoc t-test: 10 vs. 25 (0.3), 10 vs. 50 (0.02*), 25 vs. 50 (0.32); * Significance (α=0.05)



Estimated Graft Volume

Injection Location	Injection Volume: 10 μL (n=5)	Injection Volume: 25 μL (n=5)	Injection Volume: 50 μL (n=5)	ANOVA
Grey Matter	0.023 ± 0.008 mm^3	0.069 ± 0.003 mm^3	0.084 ± 0.012 mm^3	0.03[§]
White Matter	0.035 ± 0.010 mm^3	0.070 ± 0.015 mm^3	0.151 ± 0.091 mm^3	0.01[§§]
T-test	0.07	0.96	0.14	

[§] Post-hoc t-test: 10 vs. 25 (0.02*), 10 vs. 50 (<0.01*), 25 vs. 50 (0.57)
[§§] Post-hoc t-test: 10 vs. 25 (0.59), 10 vs. 50 (0.01*), 25 vs. 50 (0.08)
* Significance (α=0.05)

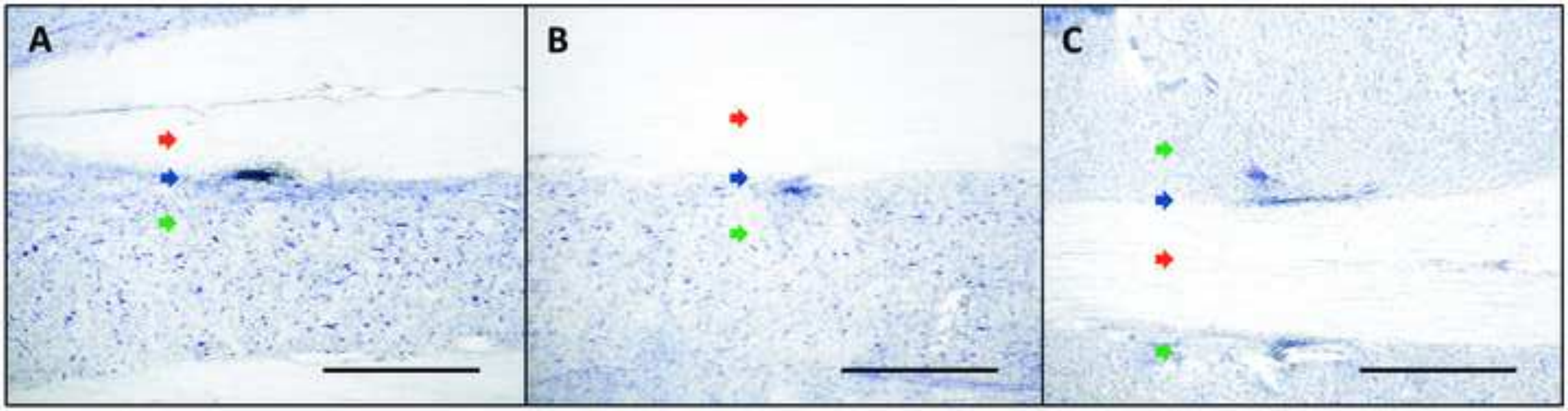

A
B
C

Table 1. Study and cell graft analysis design.

		Injection		Number of cell grafts analyzed	
	N	**Number**	**Volume**	**WM**	**GM**
Group 1	5		10 μL	5	5
Group 2	5	20 bilateral	25 μL	5	5
Group 3	5		50 μL	5	5

* WM white matter; GM grey matter

SUPPLEMENTAL MATERIALS AND METHODS

Ethics Statement

This study was carried out following the guidelines of the Institutional Animal Care and Use Committee at the University's Division of Animal Resources.

Study Design

Fifteen 12 to 18 kg healthy female Göttingen minipigs (Marshall BioResources, North Rose, NY), divided into three groups based on varying injection volumes, underwent twenty bilateral (ten on each side) injections of cells in the spinal cord. Although the ventral horn of the grey matter was targeted, injection accuracy is approximately 60% due to the lack of real-time cell tracking technologies. Therefore, each group received 10, 25, or 50 µL injections into either white or grey matter with the intent of assessing the optimal injection volume, and how escalating volume influences cell distribution in each anatomical compartment (Table1).

Cell Selection and Preparation for Transplantation

The mounting evidence provided by Clive Svendsen's laboratory in Cedars-Sinai Medical Center has supported the use of fetal-derived human neural progenitors (hNPCs) as a resource to prevent motor neuron death in ALS [1, 2]. The cells used in this study were harvested from postmortem fetal tissue (acquired by Dr. Guido Nikkhah, Germany) following approval by the Institutional Review Board at the University of Wisconsin-Madison. The resulting cell line was expanded in culture as free-floating spheres using expansion medium (Stemline Neural Stem Cell Expansion Medium, S3194; Sigma-

Aldrich, St. Louis, Missouri, USA), 100 ng/ml of human epidermal growth factor (EGF; GF003-AF, EMD Millipore, Billerica, Massachusetts, USA), and 10 ng/ml leukemia inhibitory factor (LIF; LIF1010; EMD Millipore). At passage 19, the Master Cell Bank (MCB) of hNPCs was created, stored and cryopreserved at the University's Bio-Manufacturing Facility, using current Good Manufacturing Practices. The hNPCs isolated form this MCB can grow for over 50 population doublings while keeping the potential to generate both neurons and astrocytes, but enter natural senescence between 50 and 70 population doublings [3]. The hNPCs used in this study were provided by The Svendsen Laboratory.

We received human fetal tissue derived NPCs cultured as free-floating neurospheres between passages 25 and 35. The neurospheres were cultured in maintenance medium containing human EGF, LIF, and antimicrobial/antibacterial reagent [4]. Prior to transplantation, the neurospheres were dissociated to single cells using Trypsin and DNAse [4]. The cells were then resuspended in transplantation medium with a concentration of 10,000 cells/μL and maintained on ice. The minimum accepted cell viability was >80% as determined using a hemocytometer and Trypan blue reagent.

Surgical Procedure

All animals were fasted for at least 12 hours before being anesthetized with Ketamine (35 mg/kg, IM), Acepromazine (1.1 mg/kg, IM), Atropine (0.02 mg/kg, IM), and maintained intraoperative with Isoflurane (1.5-2.5%, Inhaled) diluted in oxygen.

Multilevel Laminectomy and Cell Transplantation

In prone position, an approximate 10-15 cm incision was created over the cervical spine and a multi-level laminectomy between cervical levels 3 to 6 was performed. Following laminectomy, a stereotactic device was mounted using bilateral percutaneous posts placed through 1 cm skin incisions above and below the initial incision. As described in previous publications, the microinjection apparatus was attached to the four posts allowing it to span the laminectomy[5, 6]. The spinal cord was exposed by making a 2 to 4 cm incision into the dura mater, which was then tacked away using 4-0 Nurolon sutures (Ethicon, Inc., Somerville, NJ). The microinjection apparatus was placed and a 29-gauge custom infusion cannula was threaded through. A coordinate-based stereotactic technique was utilized to target the area of interest within the spinal cord. Ath this point methylprednisolone (Solumedrol – McKesson Corporation, San Francisco, CA) was administered as a bolus (125 mg, IV). The cannula was inserted approximately 1-2 mm medial and 4 mm deep to the dorsal root entry, and injections followed at a rate of 5 uL/minute using a microprocessor-controlled syringe pump (Microinjector – Tritech Research Inc., Los Angeles, CA). Following each infusion, the needle remained in place for an additional 1 minute to prevent retrograde cell reflux into the cannula before extraction. The needle was removed, and the injection apparatus was repositioned 4 mm away along the rostro-caudal axis to the next target site.-This technique was repeated ten times on each side of the spinal cord with respect to each injection volume cohort. After all injections were completed, the injection apparatus was removed and the incisions were closed in layers. The dura was closed using a 4-0 Nurolon stitch (Ethicon, Inc., Somerville, NJ), in a watertight fashion and the transplanted area was marked using 4-0 Prolene stitches (Ethicon, Inc., Somerville, NJ). A 0 Vicryl suture (Ethicon, Inc.,

Somerville, NJ) was used for the deep muscular layer and the muscular fascia. The dermal layer was closed with 2-0 Ethilon (Ethicon, Inc., Somerville, NJ), with a running stitch[5, 7].

Postoperative Management

On the day of surgery, a Fentanyl patch (7µg) was placed for postoperative pain management and remained in place for 72 hours. Additionally, a standard immunosuppressive regimen of intravenous (IV) Tacrolimus (Prograf, 0.025 mg/kg – Astellas Pharma US Inc, Deerfield, IL) was administered twice-daily form the day of surgery until euthanasia. Cefazolin (500 mg/day, IV) was administered starting on the day of surgery and maintained for ten days.

Euthanasia and Perfusion

Animals were sedated with Ketamine (35 mg/kg, IM), and Acepromazine (0.8 mg/kg, IM) on postoperative day 21. While cardiac function was still present, 10,000 units/ml of Heparin Sodium and Pentobarbital (0.1 ml/lb) were administered IV. Transcardiac perfusion with a peristaltic pump (Masterflex Console Drive pump model 71-1420) was carried out with a 0.9% NaCl solution followed by a 4% paraformaldehyde solution as a fixative, in order to preserve the quality of the tissue for immunohistochemistry. The region of interest (ROI) of the spinal cords was excised according to the placement of the 4-0 Prolene sutures (Ethicon, Inc., Somerville, NJ) as identifiers. The tissue collected was stored in 4% PFA for 24 hours at 4ºC, then transferred to 30% sucrose in phosphate-buffered saline (PBS) for 7 days or until tissue no longer floats. Tissue was flash frozen using 2-methylbutane and stored at -80ºC.

Histology

Frozen coronal spinal cord sections (40-50 μm thick) were cut using a cryostat (Leica CM 1950; Leica Microsystems, Nussloch, Germany). Free-floating sections were washed in PBS and blocked with horse serum (Jackson Immuno-Research) for one hour. Primary antibodies directed against the human nuclear antigen (HuNu - Millipore, catalog number MAB1281) were incubated overnight at 4ºC, and tagged with a secondary horse anti-mouse IgG (Vector Labs). Tissue was incubated overnight at 4ºC with tertiary antibodies from the ABC kit (Vector Labs) and amplified with Peroxidase Substrate (SG-black SK-4700). The tissue was then counter-stained with Cresyl Violet, mounted on slides, and cover-slipped. A similar protocol was used to detect the apoptotic marker, Caspase-3, using a human specific cleaved caspase-3 antibody at a 1:500 dilution (CellSignal, #9661) and SignalStain Boost Reagent (CellSignal, #8114). Caspase-3 slides were counterstained with using a standard protocol for eosin. All images were captured with a digital DS-Qi1 high sensitivity Cooled CCD camera using a Nikon E400 microscope supplied with NIS-Elements imaging software (Nikon Instruments, Inc.).

Graft Selection

Although the ventral horn of the grey matter was chosen as the desired target for cell transplantation, the lack of real-time cell tracking techniques makes it difficult to confirm accuracy of cell transplants during surgery. Therefore, cell grafts were ultimately distributed into both grey and white matter compartments. Representative cell grafts of each injection volume cohort in both WM (n=5) and GM (n=5) were selected randomly for analysis and grouped based on anatomical location (grey or white matter). Grafts were

excluded from analysis if any of the following were present: signs of inflammatory observation, extensive cell graft rejection and/or >50% of the graft area was damaged during tissue processing.

Microscopic Analysis

For the two-dimensional distribution analysis, serial sections were assessed and slides with the greatest expanse of HuNu+ labeled cells were chosen as the epicenter for both WM (n=5 per group) and GM (n=5 per group) cell grafts. As seen in Figure 1, rostro-caudal and transverse distribution was determined as the distance traveled by cells from the furthermost points on either side of the epicenter of the graft. Measurements were done using a length probe provided in the image processing software, Image J[8].

Stereology Protocol

Cell grafts in the WM (n=5 per group) and GM (n=5 per group) were analyzed using an unbiased uniform random sampling technique to select a representative sample (6-8 sections) for analysis. One out of every six sections was included in the sample for analysis with a total distance between sections of 300 μm. The Cavalieri method was used to estimate three-dimensional volume of each cell graft [9, 10]. The equipment used included a microscope (Leica DM2500) with a motorized x–y stage, an electronic microcator (Applied Scientific Instrumentation), which was used for measuring movements in the z direction, and the PC software (Stereologer™) for volume estimation. The sections were analyzed using a 5X objective and 60X oil-immersion objective (final magnification, 3000X). The person performing the analysis of the cell grafts was blinded to the experimental design throughout the process.

Statistical Analysis

Descriptive statistics were utilized to report outcomes and a one-way analysis of variance (ANOVA) was used to test for statistically significant differences between cohorts. Tukey's *post hoc* comparisons were used to interpret the ANOVA results. Significant differences were reported using a 0.05 confidence level.

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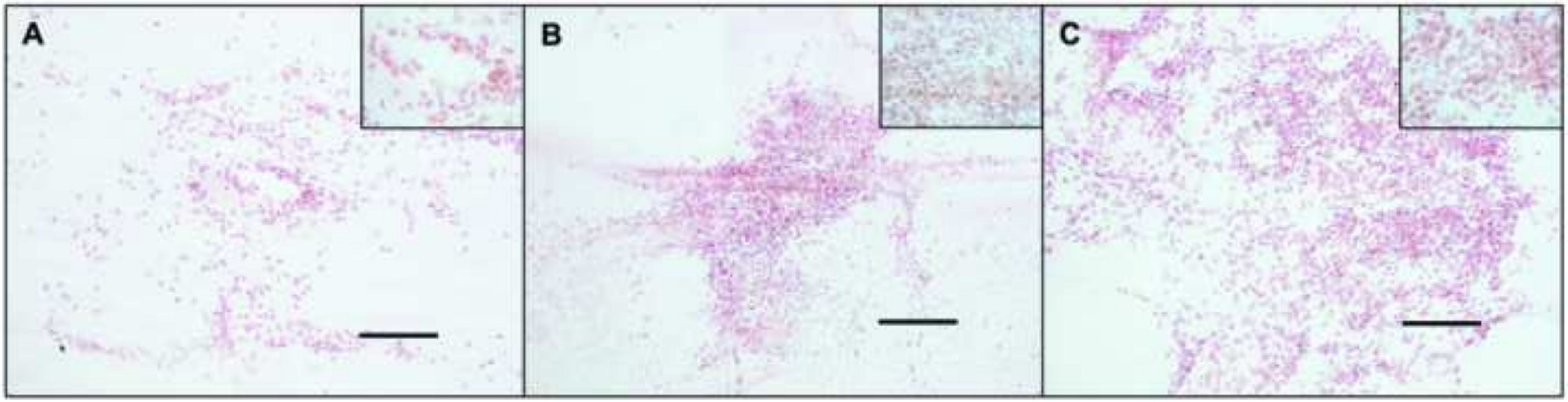
A
B
C

Peripheral Monitoring of Immune Response to Intraspinal Stem Cell Therapy

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Introduction

- Clinical investigations of intraspinal stem cell therapies are underway for a range of neurological diseases, including ALS, SCI, and MS.
- Originally considered entirely immuno-privileged, the CNS is now considered relatively privileged and immunological reactions to exogenously transplanted cell grafts have been demonstrated in mammalian models.
-From these observations, immunosuppression regimens have been employed clinically. However, graft rejection remains a significant risk and an assay to non-invasively monitor the immune response to transplanted intraspinal cell grafts is essential.

- We hypothesize that graft-specific host antibodies generated after stem cell transplantation may be detected in the peripheral blood and could be used as a diagnostic marker of cellular graft rejection.

Methods

- Large Animal Model: Ten Göttingen minipigs received five thoracolumbar intraspinal microinjections of 100,000 donor human neural progenitor cells using a stereotactic platform. Five pigs received tacrolimus (0.0125 mg/kg BID IV) and five did not receive immunosuppression. Plasma was isolated from peripheral blood collected pre-transplant and serially post-transplant at day 7, 14, and 21.

- Clinical Trial: Plasma was collected from six patients with Amyotrophic Lateral Sclerosis enrolled in the Phase 1 trial at Emory (NCT01348451) receiving intraspinal microinjections of donor human spinal cord stem cells. The patients received tacrolimus (4 - 8 ng/mL oral BID), mycophenolate mofetil (1000 mg oral BID), and basiliximab (two doses, 20 mg IV) post-operatively. Tacrolimus and mycophenolate were given for the duration of the trial. Plasma was collected pre-operatively and at post-operative week 2, 4, and 8 from three patients with naïve transplants and three patients with repeat transplants.

- Flow Cytometry Cross Match: Donor human stem cells were co-incubated with collected antibody-containing plasma from the pigs or trial patients (acting as the "primary" antibody). The cells were washed and then incubated with a FITC-conjugated secondary antibody specific to either pig or human IgG, respectively. The cell samples were washed and then run on an LSRFortessa flow cytometer. Relative mean fluorescent intensity (MFI) was measured for post-operative time points and compared to pre-operative baseline to detect the generation of graft-specific antibodies in the plasma. All time points were done in triplicate.

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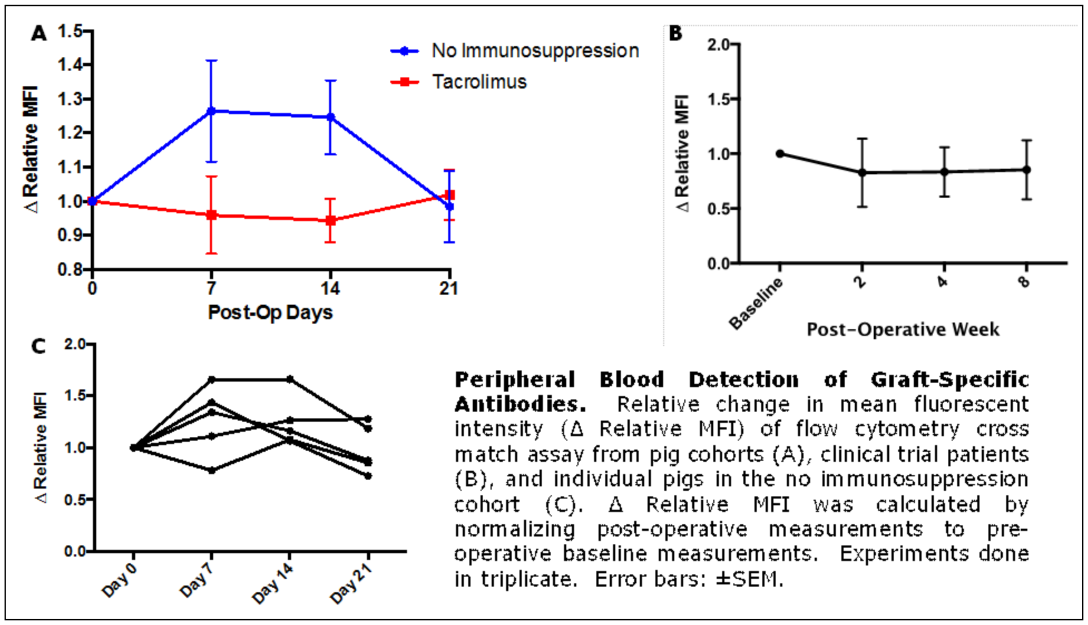

Peripheral Blood Detection of Graft-Specific Antibodies. Relative change in mean fluorescent intensity (Δ Relative MFI) of flow cytometry cross match assay from pig cohorts (A), clinical trial patients (B), and individual pigs in the no immunosuppression cohort (C). Δ Relative MFI was calculated by normalizing post-operative measurements to pre-operative baseline measurements. Experiments done in triplicate. Error bars: ±SEM.

Results

- A transient increase in graft-specific antibodies was detected 7 and 14 days post-operatively in pigs that did not receive immunosuppression. No increase was observed in the tacrolimus group (**A**).
- Different patterns of antibody response were observed in pigs in the no immunosuppression cohort, with both "responders" and "non-responders" (**C**).
- A trend showing a slight decrease in graft-specific antibodies was observed in the clinical trial patients (**B**)

Conclusions

- This method can be used to detect antibody-mediated graft rejection in vivo
- This study provides evidence for a decreased immune response to transplanted intraspinal stem cell grafts with immunosuppression
- Future studies will correlate these peripheral blood findings to immunohistological analysis of transplanted grafts

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